Drop in the Ocean: Molecular Approaches for Exploring the Diversity and Distribution of the Ascetosporea

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I certify that all material in this thesis which is not my own work has been identified and that no material has previously been submitted and approved for the award of a degree by this or any other University.

Signature:……

Abstract

The protistan class Ascetosporea (Rhizaria, Endomyxa) comprises five orders of parasites of aquatic invertebrates: Haplosporida, Paramyxida, Mikrocytida, Paradinida and Claustrosporida. The group includes a number of species known for their devastating effects as pathogens of economically significant bivalve species, the most notorious of which are the oyster pathogens *Marteilia refringens* (Paramyxida), *Bonamia ostreae* (Haplosporida) and *Mikrocytos mackini* (Mikrocytida). Due to its significance to aquaculture, interest in the group is growing, and a number of recent environmental DNA (eDNA)-based studies indicate vast, uncharacterised diversity within the class.

Traditionally species discovery and description has relied on microscopy studies of infected invertebrate tissue, and as such our understanding of the group is biased towards pathogens of commercially exploited or easily studied invertebrates, with comparatively little known about the diversity of Ascetosporea outside such hosts. Many species, particularly those infecting less “important” hosts, have not been molecularly characterised, hindering phylogenetic studies and limiting our understanding of the relationship between morphology and phylogeny.

This study uses primer-based screens of a wide range of environmental and invertebrate-associated samples to explore the diversity, distribution and host range of the four best-known ascetosporean orders, and in doing so links sequence data to described species, and allows the molecular and morphological characterisation of novel haplosporidian species in bivalve hosts.

PCR screens also uncovered a large radiation of novel sequence types within Paradinida, in littoral and coastal environmental samples, and demonstrated the abundance and diversity of haplosporidians in freshwater and terrestrial sample types.

This study also focuses on the development and application of sensitive complementary molecular and microscopy-based methods for the detection of parasite life-stages in host tissues, and consideration given to the role of molecular methods in facilitating rapid, accurate diagnosis of pathogens in important hosts.

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Ward GM, Bennett M, Bateman KS, Stentiford GD, Kerr R, Feist SW, Williams ST, Berney C, Bass D. 2016. A new phylogeny and environmental DNA insight into paramyxids: an increasingly important but enigmatic clade of protistan parasites of marine invertebrates. Int. J. Parasitol. 46(10): 605-619.

Ward GM, Neuhauser S, Groben R, Ciaghi S, Berney C, Romac S, Bass D. 2018. Environmental sequencing fills the gap between parasitic haplosporidian and free-living giant amoebae. J. Eukaryot. Microbiol. doi: 10.1111/jeu.12501.

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Hartikainen H, Stentiford GD, Bateman KS, Berney C, Feist SW, Longshaw M, Okamura B, Stone D, **Ward GM**, Wood C, Bass, D. 2014b. Mikrocytids are a broadly distributed and divergent radiation of parasites in aquatic invertebrates. Curr. Biol. 24: 807-813.

Kerr R, **Ward GM**, Stentiford GD, Alfjorden A, Mortensen S, Bignell JP, Feist SW, Villalba A, Carballal MJ, Cao A, Arzul I, Ryder D, Bass D. 2018. *Marteilia refringens* and *Marteilia pararefringens* sp. nov. are distinct parasites of bivalves and have different European distributions. Parasitology 145(11): 1483-1492.

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Introduction

Seafood (including finfish, Crustacea, bivalves and, increasingly, algae) is the most highly traded food commodity (Stentiford et al., 2017). As aquaculture expands to meet rising demand, diseases caused by parasites have a significant impact on the sustainability and economic stability of the industry (Shinn et al., 2015).

While a number of aquaculture pathogens are well-established in farmed populations and their effects well known, incidence of mortality caused by emerging disease continues occur across all aquaculture sectors. Emerging diseases are those caused by a pathogen which has not previously been observed, or where organism's role as a disease-causing agent has not previously been recognised. The appearance of a known disease agent in a new geographic location, host population, or a new host may also be considered emerging disease (Moutou & Pastoret, 2015). This emergence may be the result of the introduction of the pathogen to a population by natural means (e.g. a change in environmental conditions expanding the geographic range of vector species or intermediate hosts), or human-mediated, for example as a result of translocation of stocks between geographic regions.

The World Organisation for Animal Health (OIE) produces an annual list of diseases, infections and infestations considered to be specific threats to terrestrial or aquatic animal health. Incidence of any listed disease must be reported to the OIE, and affected populations may be destroyed or subject to

national or international trade restrictions. As of 2019, the OIE lists ten diseases in finfish (all but one of which are viruses), nine infecting crustaceans (6 viruses, 1 bacteria and 1 protist). Seven mollusc diseases are listed: one virus (abalone herpesvirus), one prokaryote (*Xenohaliotis californiensis*, infecting *Haliotis* spp. abalone), and five protists. Among these are alveolate diseases of oysters and clams (*Perkinsus marinus* and *P. olseni*), and three parasites belonging to the Ascetosporea (*Bonamia ostreae, B. exitiosa* and *Marteilia refringens*), all of which are pathogens of oysters (OIE, 2019).

The protistan infrakingdom Rhizaria includes a number of significant parasites, split into two assemblages: the Phytomyxea, obligate parasites of plants, diatoms and brown algae (Neuhauser et al., 2014), and the Ascetosporea, infecting aquatic invertebrates including molluscs, crustaceans and annelid worms, known from both freshwater and marine environments (Cavalier-Smith 2002; Sierra et al., 2016). Phytomyxea and Ascetosporea have previously considered to be members of Endomyxa, along with the free-living amoebae *Gromia* and *Filoreta*), based on 18S rRNA phylogenies (Cavalier-Smith, 2002). More recent phylogenomic analyses including three ascetosporean lineages (mikrocytid *Mikrocytos mackini* and haplosporidians *Minchinia chitonis* and *Bonamia ostreae*) show Ascetosporea to group as sister to Apofilosa (*Gromia* + *Filoreta),* with Ascetosporea + Apofilosa together sister to Retaria (Sierra et al., 2016; Fig. Int-01).

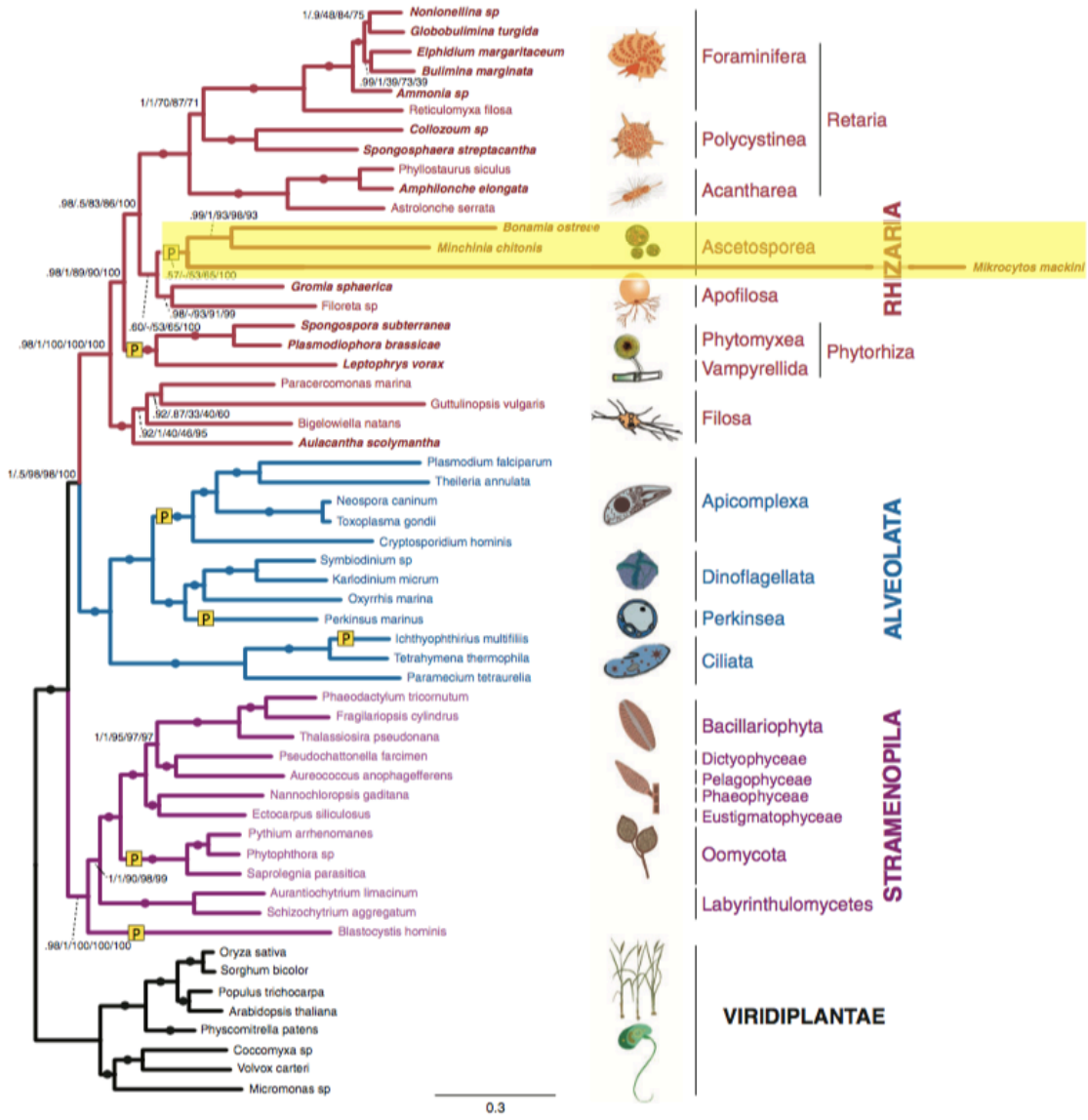

Figure Int-01. Bayesian phylogeny of the SAR (Stramenopila, Alveolata, Rhizaria) group from Sierra et al. (2016), based on 229 genes. The position of the Ascetosporea within Rhizaria is highlighted within the yellow box.

Ascetosporea comprises five orders: Haplosporida, Paramyxida, Mikrocytida, Paradinida and Claustrosporida (Bass et al., 2009). Members of all five orders infect aquatic invertebrates. Haplosporida, Paramyxida and Mikrocytida all include notorious pathogens of economically significant oyster species, including *Haplosporidium nelsoni* and *Bonamia ostreae* (Haplosporida), *Marteilia refringens* and *M. sydneyi* (Paramyxida) and *Mikrocytos mackini* (Mikrocytida). Paradinida and Claustrosporida are much less well known. Paradinida is comprised of a small number of *Paradinium* spp. marine copepod

parasites, and Claustrosporida was erected on the basis of scant morphological evidence of two freshwater crustacean parasites of the genus *Claustrosporidium* (Bass et al., 2009; Larsson 1987), for which there are no molecular data available.

Due to the effects of many ascetosporean species on commercially exploited hosts, certain species within the group have received a lot of attention. Both *Marteilia refringens* and *Bonamia ostreae* are responsible for significant mortalities in the flat oyster *Ostrea edulis* in Europe, and so both are listed as notifiable pathogens in World Organisation of Animal Health (OIE) and European Union legislature, restricting trade between areas where either disease is known to occur and those considered free of disease. *M. refringens* emerged as a pathogen of *O. edulis* in France in the late 1960s (Comps, 1970), and losses attributed to this parasite were compounded by the appearance of *B. ostreae* in the 1970s (Engelsma et al., 2014). The impact of both parasites combined resulted in the catastrophic decline of flat oyster culture in Europe. The flat oyster industry has not recovered (Carrasco et al., 2015), and the non-native Pacific oyster *Crassostrea gigas* is now favoured in culture in Europe and elsewhere for its fast growth rate, large size (Andrews, 1980) and apparent resistance to a number of diseases, including both *M. refringens* and *B. ostreae* (Renault et al., 1995), and *Perkinsus marinus* (Calvo et al., 1999).

C. gigas is, however, susceptible to a number of other ascetosporean species. Significant economic losses have been attributed to the microcell *Mikrocytos mackini* across only a relatively small geographic area along the west coast of North America (Garcia et al., 2018) and another mikrocytid, *M. mimicus*, was

recently described causing localised mortalities in *C. gigas* cultured in Norfolk, UK (Hartikainen et al., 2014b). The paramyxid *Marteilia (Marteilioides) chungmuensis* is known to cause mortalities in *C. gigas* in South Korea and Japan, and those which survive infection are left with nodules on the gonad, which significantly reduces their market value (Itoh et al., 2003b).

Comparatively much less is known about species not known to infect commercially exploited hosts, and the distribution of Ascetosporea in areas where there is little aquaculture activity or dedicated research effort is largely unexplored. The paucity of fully characterised species in several orders, particularly Mikrocytida and Paradinida, means the extent of the genetic and morphological diversity within each order is largely unknown, as is the host range of many species, and no complete life cycle is known for any ascetosporean lineage. The involvement of intermediate hosts is suspected in the life cycle of many species, including *Haplosporidium nelsoni* (Ford & Bushek, 2012) and *Marteilia refringens* (Audemard et al., 2002), however the identity of these intermediate hosts are largely unknown, as are potential vector species or environmental reservoirs of pathogens (Berthe et al., 2004; Lynch et al., 2010).

In recent years, a number of studies utilising environmental DNA (eDNA) techniques have hinted at significant uncharacterised 18S rRNA sequence diversity within Ascetosporea (Hartikainen et al., 2014a,b; Pagenkopp-Lohan et al., 2016), particularly Haplosporida. eDNA approaches use DNA (or RNA transcribed into cDNA) from environmental samples, such as sediments, water, soil or air (Bass et al., 2015). The nucleic acids contained within these samples

include those derived from living organisms within the sample, but it is important to consider too that extracellular DNA – DNA not contained within cells, having been released either by cell lysis or by active secretion (Nagler et al. 2018) – is present in all nearly environmental samples.

eDNA surveys, particularly when paired with high-throughput sequencing technologies, are a powerful tool for the detection of genetic diversity, including surveys of microbial diversity in extreme environments, and are increasingly employed in parasitology for the detection of parasite DNA outside of the host. This presence of parasite DNA in environmental samples may represent living parasite life-stages, such as spores, eggs or infective stages, or extracellular DNA, and so caution must be used when inferring the presence of a parasite from eDNA surveys alone (Bass et al., 2015).

Haplosporida

The most well-studied ascetosporean order is the Haplosporida, which comprises over 40 formally described species and a number of as-yet unnamed species which have not been fully characterised (Arzul & Carnegie, 2015). The order has also been shown by recent environmental and host-targeted molecular studies to harbour significant uncharacterised sequence diversity (Hartikainen et al., 2014a; Pagenkopp-Lohan et al., 2016). Within the order there are four genera: *Haplosporidium*, parasites of annelid worms, molluscs and crustaceans in marine and freshwater environments (Arzul & Carnegie, 2015), *Minchinia*, infecting marine molluscs including bivalves and chitons (Ford et al., 2009), *Urosporidium*, known largely as hyperparasites in trematode worms but also infecting marine free-living annelids (Burreson & Ford, 2004),

and *Bonamia*, a group of “microcell” parasites infecting a number of oyster species (Engelsma et al., 2014).

The best known haplosporidian species include *Haplosporidium nelsoni*, pathogen of the eastern oyster *Crassostrea virginica* in the United States of America, and *Bonamia ostreae*, which is a major cause of mortality in flat oysters *Ostrea edulis* in Europe.

H. nelsoni, also known as MSX (‘multinucleated sphere X’), was first introduced to the Delaware Bay on the mid-Atlantic coast of the USA before 1957, when the parasite began causing mortalities in *C. virginica* populations (Carnegie & Burreson, 2011). In 1959 *H. nelsoni* spread to oysters in the nearby Chesapeake Bay, and within two years had killed more than 90% of oysters in high-salinity regions within these two affected areas (Burreson et al., 2000). The parasite continued to impact oyster populations in the region over the next 20 years until the 1980s, when *Perkinsus marinus* emerged as the dominant pathogen in this region (Carnegie & Burreson, 2011). The incidence of *H. nelsoni* in *Crassostrea* spp. in the region is now much lower, and not associated with catastrophic mortalities on the same scale (Ford et al., 2009; Carnegie & Burreson, 2011). The exact cause of this apparent decline in the virulence of *H. nelsoni* in these areas is not known, and may be the result of changes in environmental conditions, such as milder winter temperatures or changes in the benthic community structure (i.e. reservoir species or intermediate hosts), but is thought to be at least partly due to increased host resistance to the parasite (Carnegie & Burreson, 2011).

Bonamia ostreae, which emerged as a parasite of the flat oyster *Ostrea edulis* in Europe shortly in the late 1970s, is one of two ascetosporean species responsible for the dramatic decline of the *O. edulis* culture industry in Europe (the second species, paramyxid *Marteilia refringens*, is discussed in the following section). Unlike *H. nelsoni*, the impact of *B. ostreae* has not abated, and as such the pathogen is still listed under OIE and EU legislation in order to control the movement of potentially infected stocks and mitigate losses associated with the parasite (OIE, 2018).

Though the life cycles of all ascetosporean species remain unknown, *H. nelsoni* and *B. ostreae* are suspected to use very different transmission strategies. *B. ostreae*, which is not known to form spores, has been shown to be directly transmissible between flat oyster hosts via water currents under laboratory conditions (Culloty et al., 1999; Montes et al., 2004), whereas experimental transmission of *H. nelsoni* between hosts *C. virginica* has proven unsuccessful (Andrews, 1979) and so an alternative host is widely suspected though as yet unidentified (Powell et al., 1999).

Over 40 haplosporidian species have been described, however sequence data is only available for a small number of these. The majority of these species were described before the advent of modern molecular methods, and so description relied on the observation of ultrastructural characteristics by microscopy. As the plasmodial stages of haplosporidians are all morphologically very similar, spore structure has traditionally been used, often inconsistently, for the identification of haplosporidians to genus or species level.

Perkins (2000) proposed the description of haplosporidian species should be on the basis of spore ornamentation, with *Bonamia* species at that time presumed not to form spores. Haplosporidians form ovoid, walled spores which have an orifice at one pole, and which lack polar filaments or tubules. In the classification system put forward by Perkins (2000), spores of *Haplosporidium* and *Minchinia* species have an external lid covering the orifice, whereas *Urosporidium* spp. spores have an internal flap of cell wall material forming a lid. The spores of *Urosporidium* species are unornamented, while both *Haplosporidium* and *Michninia* form ornamented spores. Species in which this ornamentation is visible by light microscopy Perkins (2000) assigned to *Minchinia*, and those in which it is not to *Haplosporidium* (regardless of the origin of spore ornamentation). This classification system did not gather wide support, with most workers preferring the classification system previously put forward by Ormières (1980): species in which spore ornamentation is composed of epispore cytoplasm should be placed in *Minchinia*, while those whose spore ornamentation originates from cell wall material should be placed in *Haplosporidium* (Burreson & Ford, 2004).

While these criteria seem to hold for all described *Minchinia* and *Urosporidium* species, *Bonamia perspora*, the only species within the genus known to form spores, produces spores matching those criteria for *Haplosporidium* spp. (Carnegie et al., 2006). *Bonamia* groups consistently and robustly as sister to *Minchinia* in phylogenetic analyses (Burreson & Reece, 2006; Hartikainen et al., 2014a; Arzul & Carnegie, 2015), and so haplosporidians with '*Haplosporidium*' spore structures do not form a monophyletic group. The use of these ultrastructural characteristics for assigning species to genera are therefore

unsupported, particularly as spore ornamentation for the type species of *Haplosporidium, H. scolopli*, is unknown (Burreson & Reece, 2006).

Reliance on spore structure to allow the formal description of haplosporidians has resulted in a long list of uncharacterised species (Arzul & Carnegie, 2015), with some known only from incomplete ultrastructural data (e.g. Comps & Tigé, 1997; Carballal et al., 2005), while others may have both morphological and molecular data available and yet still remain uncharacterised due to a lack of spore morphology (e.g. Utari et al., 2012). There also exist a large number of haplosporidian sequence types for which no morphological data is available, but seemingly prevalent in marine environmental samples (Hartikainen et al., 2014a).

Paramyxida

The taxonomy of the order Paramyxida Chatton 1911 has long been confused, and has been subject to revision a number of times (Desportes 1984; Desportes & Perkins, 1990; Feist et al., 2009). Most recently, following years of the inconsistent use of different ultrastructural characteristics as diagnostic criteria for each genera, Feist et al. (2009) revised the order and proposed that the nature of the spore be used as the defining characteristic for assignation of species to genera: those producing bicellular spores belong to the genus *Paramarteilia*, species in which spores are tricellular belong to *Marteilia*, and species producing tetracellular spores should be assigned to *Paramyxa.* In doing so, Feist et al. (2009) suppressed the genera *Marteilioides* (with *M. chungmuensis* transferred to *Marteilia* and *M. branchialis* transferred to *Paramarteilia*), and *Paramyxoides* (with *P. nephtys* transferred to *Paramyxa*).

Species within the genera *Marteilia* are parasites of marine bivalves, and include species responsible for mass mortalities in infected oysters (*M. refringens* in *Ostrea edulis* Europe; *M. sydneyi* in *Saccostrea glomerata* in Western Australia), or producing unsightly lesions in host tissues and therefore reducing their market value (*M. chungmuensis* in *Crassostrea gigas* in Japan) (Berthe et al., 2004; Carrasco et al., 2015). *Paramarteilia* spp. are parasites of crustaceans, including the amphipod *Orchestia gammarellus* (*P. orchestiae*; Ginsburger-Vogel & Desportes, 1979*)*, edible crab *Cancer pagurus* (*P. canceri*; Feist et al., 2009) and European spider crab *Maja squinado* (*Paramarteilia* sp.; Feist et al., 2009), with a single species, *P. branchialis* infecting a bivalve host (oyster *Saccostrea glomerata*). *Paramyxa* comprises two species, both parasites of marine annelid worms. The type species of the genus, *P. paradoxa*, was described from polychaete larvae collected on the Mediterranean coast of France (Chatton, 1911), and remained the only species within the genus until the discovery of *P. nephtys* infecting *Nephtys caeca* almost 100 years later (Larsson & Køie, 2005).

While molecular data are increasingly available for paramyxids, the majority of sequence data generated is for a small number of species, particularly *Marteilia* spp. from bivalves in Europe. Only a single sequence type tentatively linked to *Paramarteilia* from amphipod host *Echinogammarus marinus* was available until very recently (Short et al., 2012a), with no sequence data available for any *Paramarteilia* parasite of crabs, or *P. branchialis*. No sequence data are available for either *Paramyxa* species. Phylogenetic analysis by Short et al. (2012a), including 18S sequence data for *M. refringens, M. chungmuensis* and

Paramarteilia sp. of amphipods do not support the recent revision of the order by Feist et al. (2009), showing instead *M. chungmuensis* groups as sister to *Paramarteilia* with strong support, with *M. refringens* basal to this pairing. However it is important to note the small number of taxa included in this analysis. Given the importance of paramyxid parasites it is a priority and therefore one of the aims of this thesis to increase the availability of sequence data for the order, particularly the molecular characterisation of species and genera for which sequence data is not available. Equally as important is pairing this sequence data unequivocally with the morphology of the parasite, in order to rationalise the nomenclature of the group.

PCR screens have proven successful for the orders Haplosporida (Hartikainen et al., 2014a) and Mikrocytida (Hartikainen et al., 2014b) in uncovering novel sequence diversity and for the detection of known lineages within environmental samples and invertebrate tissues, giving insight into the ecology of these parasites and their potential host range. The same principles will be applied in this study for Paramyxida, with the aim of expanding our knowledge of the order beyond a handful of commercially important parasites and understanding the global distribution of the group.

It is important too to deepen our understanding of paramyxid parasites affecting commercially important species. *Marteilia refringens* is a pathogen of the flat oyster *Ostrea edulis*, but is also able to infect a number of other bivalve species in Europe including mussels *Mytilus edulis*, *M. galloprovincialis* and *Xenostrobus securis* (Comps 1975; Comps 1982; Pascual et al., 2010), and

clams *Chamelea gallina, Solen marginatus* and *Ruditapes decussatus* (Lopes-Flores et al., 2008a, b; Boyer et al., 2013).

The history of *Marteilia* species infecting *Mytilus* spp. is somewhat confused. While *M. refringens* has been observed infecting mussels in Spain and France since shortly after the parasite emerged in oysters in the late 1960s (Tigé & Rabouin, 1976; Comps & Joly 1980; Villalba et al., 1993), Comps et al. (1982) described a very closely related species, *M. maurini*, infecting *M. galloprovincialis* imported to France from Venice lagoon, Italy. Though morphologically incredibly similar, this parasite was discriminated from *M. refringens* on the basis of subtle ultrastructural characteristics and supposed host preference (Comps et al., 1982; Villalba et al., 1993). The identity of *Marteilia* species infecting mussels has since remained a contentious subject. Le Roux et al. (1999) showed *M. refringens* and *M. maurini* to have identical small subunit rRNA gene sequences, and subsequent examination of the ultrastructural characteristics of a large number of examples of *Marteilia* parasites in *O. edulis* and *Mytilus* spp. concluded that there was no basis to the distinction between the two species, and so they were synonymised (as *M. refringens*) (Longshaw et al., 2001; Balseiro et al., 2007). The synonymisation of these two species was not without consequence. As a result of the pathogen's effects on *O. edulis* populations across Europe, *M. refringens* is listed as a notifiable disease under both OIE and EU legislature, and so now all incidences of *Marteilia refringens* in mussels and oysters must now be subject to the trade restrictions imposed following discovery of the parasite.

Sequencing of the faster-evolving ribosomal first internal transcribed spacer (ITS1) region of *Marteilia refringens* revealed two distinct sequence 'types' (Le Roux et al., 2001). However despite these types being labelled 'O' (oyster) and 'M' (mussel), to indicate their supposed host preference, both types are in fact able to infect both hosts, and co-infections of both types have been reported (Lópes-Flores et al., 2004; Novoa et al., 2005).

M. refringens M-type is only sequence type to have been detected to date in northern Europe, where it has only been observed infecting *Mytilus* spp. with no reported mass mortality events attributed to the parasite. *M. refringens* (of either 'type') has never been reported infecting *O. edulis* in the UK (Laing et al., 2014), or in other northern European country where the parasite has been observed in mussels (Swedish Veterinary Institute, unpublished data). An important aim of this study and complementary studies (see Appendix II) is to establish the presence of and distribution of these two genotypes within bivalve populations in northern Europe, and gain a greater understanding of the differences between the two *M. refringens* types infecting bivalves in Europe. These types are often treated as a single entity, and so the differences between the pathogenicity and prevalence of *Marteilia* in different bivalve hosts and populations (particularly those in which no mortality events have been known to occur) are largely unknown and unexplored.

Mikrocytida

Mikrocytids are "microcell" parasites of aquatic invertebrates, characterised by their very small (<5 µm) cell size (Carnegie & Cochennec-Laureau, 2004). Mikrocytids also lack most organelles found in eukaryotic cells including

mitochondria (Hine et al., 2001), though phylogenomic analysis of *Mikrocytos mackini* by Burki et al. (2013) confirmed the parasite possesses mitochondrion-derived genes. Superficially *M. mackini* resembles *Bonamia* spp. at light microscope level (Carnegie & Cochennec-Laureau, 2004), however ultrastructural differences, particularly the lack of mitochondria and the haplosporosomes observed in *Bonamia* and other haplosporidians (Hine et al., 2001), as well as greatly divergence between their 18S rDNA sequences, ruled out any close affiliation between *Mikrocytos* and Haplosporida.

The taxonomic placement of mikrocytids was unknown for many years until recent phylogenomic analyses confirmed an affiliation between *Mikrocytos*, its recently described sister taxon *Paramikrocytos*, and the Haplosporida, and the order Mikrocytida was erected within Ascetosporea (Hartikainen et al., 2014b; Sierra et al., 2015).

The best known mikrocytid species is *Mikrocytos mackini*, the causative agent of Denman Island disease, a major cause of mortality in the Pacific oyster *Crassostrea gigas* on the Pacific coast of Canada. Until very recently *M. mackini* was the only described mikrocytid species, though a number of species have been characterised in the past few years: *Mikrocytos boweri*, parasite of Olympia oysters *Ostrea lurida* in Canada (Bower et al., 2014a), *M. mimicus*, parasite of *C. gigas* in Norfolk, UK (Hartikainen et al., 2014b), and *Paramikrocytos canceri*, a parasite of juvenile edible crabs *Cancer pagurus* in the southwest UK and the only mikrocytid to date shown to infect crustaceans (Hartikainen et al., 2014b). A handful of uncharacterised species have been observed infecting flat oysters *Ostrea edulis* on the Atlantic coast of Canada

(Gagné et al., 2008), clams *Donax trunculus* in France (Garcia et al., 2012) and *Ruditapes philippinarum* in Spain (Ramilo et al., 2014).

Most observed mikrocytid lineages have not been formally described due to a lack of taxonomically relevant ultrastructural characteristics (Abbot & Meyer, 2014), and a recent study by Hartikainen et al. (2014b) (see Appendix II) revealed 10 novel mikrocytid sequence types across three novel clades and two described genera in environmental and invertebrate sample types, suggesting the existence of significant, as-yet uncharacterised diversity within the order.

Mikrocytids are emerging diseases in important aquaculture hosts, and the diversity and geographic distribution of the group is only just beginning to come to light. One of the aims of this study is to expand upon the group-specific PCR probing of Hartikainen et al. (2014b) to include the targeted screening of sample types associated with potential hosts of commercial significance, and samples from a wider range of geographic regions. During the course of this study sequence data became available for two further novel mikrocytid species, *Mikrocytos donaxi* and *M. veneroïdes* infecting *Donax trunculus* in France (Garcia et al., 2018). These data, and data for other novel lineages discovered as part of this study, did not overlap with the majority of existing mikrocytid sequence data, and were too divergent to be detected using the nested, mikrocytid-specific primer set of Hartikainen et al. (2014b).

Paradinida

Paradinida is a little-studied order whose affiliations with the Ascetosporea have also only recently come to light. The only described genus *Paradinium* is

comprised of three species of marine copepod parasites, with Chatton (1920) originally suggesting an affinity between this group and the Dinozoa or syndinians based on their morphology. However availability of 18S sequence data for *Paradinium poucheti* (Skovgaard & Daugbjerg, 2008) and a so-called 'haplosporidian' parasite of the spot prawn *Pandalus platyceros* (dubbed SPP) (Bower & Meyer, 2002) showed these taxa to group with an uncharacterised environmental clade, ENDO-3, as sister to Haplosporida, and the order Paradinida was erected by Cavalier-Smith (Bass et al., 2009). Also within this clade are the giant testate amoeba *Gromia*, and the reticulate amoeba *Filoreta*.

Both morphological and sequence data are lacking for Paradinida, with sequence data available only for one of the three described species of *Paradinium*, and a small number of other uncharacterised lineages within the group, for which no morphological data is available. Copepods are the most abundant metazoans in marine plankton (Turner, 2004), however the effects of parasitism on populations is largely unknown (Skovgaard & Saiz, 2006).

Claustrosporida

The final ascetosporean order, Claustrosporida, was originally erected as the family Claustrosporidiidae within Haplosporida (Larsson 1987). The family contained a single genus, *Claustrosporidium*, comprising two species, *C. gammari* and *C. asellii*. Both were originally described within the genus *Haplosporidium* (Ryckeghem, 1930; Pflugfelder, 1948), but were reassigned to *Claustrosporidium* on the basis that they form much simpler spores than *Haplosporidium* spp., lacking an orifice, lid structure and the ornamentation characteristics used as definitive for assignation of haplosporidian species to

genera (Larsson 1987). Claustrosporidiidae was raised to the rank of order by Cavalier-Smith & Chao (2003) during revision of the subphylum Endomyxa. No molecular data exist for either *C. gammari* or *C. asellii*, and no other members of the order have yet been identified. Use of spore structures as diagnostic criteria for taxonomy has since proven to be invalid within Haplosporida, and with no sequence data and limited ultrastructural characteristics available for the order, the affinities of Claustrosporida remain unknown.

While Claustrosporida itself does not form a chapter in its own right in this thesis, the diversity of haplosporidians in freshwater and terrestrial environments and potential invertebrate hosts is explored, including PCR screens of *Gammarus pulex* and *Asellus aquaticus*, hosts of *Claustrosporidium* spp. Haplosporidian species are known to infect other freshwater invertebrates, including snails (*Haplosporidium pickfordi*) and mussels (*H. raabei*), however comparatively little is known of the diversity of the group outside of marine environments. A deeper understanding of the extent of haplosporidian freshwater diversity within both environmental samples (as has proven successful in marine environments; Hartikainen et al., 2014a) and potential host tissues, will offer insight into the potential host range of these lineages, with a view to further characterising this diversity with complementary microscopic data in future studies.

An overarching aim of this thesis is to gain a greater insight into the diversity of 18S rRNA sequences for all ascetosporean orders. Previous studies targeting haplosporidians and mikrocytids have shown eDNA surveys to be effective tools for the detection of novel sequence diversity with marine water and sediment

(Hartikainen et al., 2014a,b), and so it may be hypothesised that adapting these approaches, which utilized targeted primer sets based on known diversity, will reveal similar findings for the less studied orders, namely Paramyxida and Paradinida, as shown in chapters One and Four respectively.

Similarly eDNA surveys focusing on Haplosporida have focused almost entirely on marine environments, despite haplosporidian species also being known from freshwater hosts. It is expected therefore that extending techniques previously employed in marine environments and tailoring them to non marine sample types will reveal further novel sequence types and novel clades, as presented in Chapter Five.

Since work on this thesis began, our knowledge of the Mikrocytida has improved significantly, with two distinct novel species described from wedge clams *Donax trunculus* in France (Garcia et al., 2018). Chapter Six aims to address disparities in available sequence data for characterised mikrocytid species, and provide overlapping 18S sequence data for as many lineages as possible. This in turn will allow the generation of more robust phylogenies with highest possible taxon sampling problem, with a view to facilitating the design of more inclusive group-targeted primers for the continuation of mikrocytid research.

The development of group-targeted primer sets is a valuable tool for the detection of both known and unknown diversity, in environmental and organismal samples. Both molecular and morphological data are reqired to understand the evolutionary relationships between lineages, and providing

complementary data for as many ascetosporean lineages as possible will lead to greater insight into the group, their diversity and their life cycles, as well as facilitating the development of robust and rapid diagnostic methods for their detection. As such this work also focused on providing molecular data for lineages to date known only from morphology-based studies, namely the paramyxids *Paramyxa nephtys* and *Paramarteilia orchestiae*. These genera are both poorly sampled molecularly, and so Chapter One demonstrates the efficacy of a targeted sampling approach, paired with complementary PCR, histopathology and *in situ* hybridisation for the complete characterisation of parasite lineages.

Chapter Two focuses on the application of complementary molecular and histopathological techniques for the detection and characterisation of haplosporidian parasites in the blue mussel *Mytilus edulis* in the UK. Blue mussels are an important aquaculture commodity in Europe, and there have been several previous reports of haplosporidians infecting these species and close relatives. However little is known about the prevalence of these parasites or their significance as pathogens, and so this work aims to characterise observations of haplosporidian parasites in both wild and exploited populations of *M. edulis*.

An important aspect of disease management in marine environment is the development of consistent and reliable tools for the detection of pathogens. The uniform application of these protocols aims to mitigate and localise disease outbreaks and prevent the spread of pathogens. Many protocols for the detection of the disease rely on histopathological methods, which in turn rely on

trained, experienced technicians. Time constraints often mean that only a small number, in many cases only a single slide, can be examined per individual. Chapter Three employs a range of increasingly sensitive molecular approaches, including DNA and RNA templates, paired with diagnostic light microscopy protocols for the detection of *Marteilia* in *Mytilus edulis* in the UK. The study site in the Tamar estuary, Devon, is the only location in England where *Marteilia* is currently known to be detectable, and it is considered to be a low prevalence parasite not currently causing mortalities. By applying such methods, this study aims to determine whether the true prevalence of the parasite at this site is underestimated, and whether molecular-led diagnosis protocols offer a more sensitive and rapid option for disease detection.

Paradinida is the least studied of the four ascetosporean orders covered in this thesis, largely because they are not currently known to infect commercially important hosts, with the exception of a single, uncharacterised parasite of the spot prawn (Reece, 2000). All described species within the order infect copepods, and so sequence types grouping with known paradinids may be expected to be prevalent within littoral, coastal and deep-sea environmental samples, where copepods play an important role in marine food webs. Chapter Four employs a targeted PCR approach similar to those applied to other ascetosporean orders, paired with the mining of ascetosporean-like sequence types from high-throughput sequence datasets from littoral, coastal and deep-sea water and sediment samples, as well as guts of bivalves, to offer insight into the 18S sequence diversity of the order, and diversity at the base of Ascetosporea, between the parasitic haplosporidians and their closest known characterised relatives *Gromia* and *Filoreta.*

Chapter One:

A New Phylogeny and Environmental DNA Insight into Paramyxids: An Increasingly Important but Enigmatic Clade of Protistan Parasites of Marine Invertebrates

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Chapter Description:

This chapter is focused on summarising the problems which have restrained paramyxid research to date, particularly the inconsistent application of ultrastructural characteristics to taxonomic assignation, and a similar lack of consistency in the generation of molecular data for both previously described and novel taxa. The formation of the 'Paramyxean Working Group' in early 2015, comprised of researchers from Spain, France, the UK, Australia and South Korea demonstrates a growing awareness of the importance of paramyxid parasites in countries where bivalve aquaculture is economically significant. The group calls for an international collaborative approach to paramyxid research, as well as more consistent application of nomenclatural terms and ultrastructural description.

The chapter also required a thorough literature search and the critical appraisal in each case of reports of paramyxid and paramyxid-like parasites in novel hosts or geographic locations since their emergence as important diseases relevant to aquaculture in the 1960s. For the purposes of this chapter, all reports which were unclear, unreliable or have since been found to be erroneous have been excluded, to provide a robust, comprehensive summary of paramyxid research to date.

An important outcome of this chapter is the presentation of the most complete molecular phylogeny of paramyxid 18S rRNA sequence data to date, including sequence data generated from environmental and organismal samples and collated from the publicly available NCBI GenBank database. This builds upon

similar studies focusing on the diversity and ecological partitioning of other ascetosporean orders, namely the Haplosporida and Mikrocytida (Hartikainen et al., 2014a,b). There was a focused effort on producing 18S sequence data for lineages which have until now been known only from ultrastructural studies, particularly species within the genera *Paramyxa* and *Paramarteilia*. This not only strengthens phylogenetic analyses and increases our understanding of paramyxid ribosomal small subunit sequence diversity, but also facilitates more complete species descriptions within the order.

Author's Contribution:

Georgia Ward contributed to the conception and experimental design, as well as collection and processing of environmental and invertebrate samples. She designed and optimised both sets of paramyxid group-specific primers, and designed and utilised DIG-labelled *in situ* hybridisation probes for both *Paramyxa nephtys* and *Paramarteilia orchestiae*. She performed all phylogenetic analyses, and was heavily involved in drafting and revising the manuscript for publication.

Abstract

Paramyxida is an order of rhizarian protists that parasitise marine molluscs, annelids and crustaceans. They include notifiable pathogens (*Marteilia* spp.) of bivalves and other taxa of economic significance for shellfish production. The diversity of paramyxids is poorly known, particularly outside of commercially important hosts, and their phylogenetic position is unclear due to their extremely genetically divergent 18S rDNA sequences. However, novel paramyxean lineages are increasingly being detected in a wide range of invertebrate hosts, and interest in the group is growing, marked by the first 'Paramyxean Working Group' meeting held in Spain in February 2015. We review the diversity, host affiliations and geographical ranges of all known paramyxids, present a comprehensive phylogeny of the order and clarify its taxonomy. Our phylogenetic analyses confirm the separate status of four genera: *Paramarteilia, Marteilioides, Paramyxa* and *Marteilia*. Further, as including *M. granula* in *Marteilia* would make the genus paraphyletic we suggest transferring this species to a new genus, *Eomarteilia*. We present sequence data for *Paramyxa nephtys* n. comb., a parasite of polychaete worms, providing morphological data for a clade of otherwise environmental sequences, sister to *Marteilioides*. Light and electron microscopy analyses show strong similarities between both *Paramyxa* and *Paramyxoides*, and we further discuss the validity of these two genera. We provide histological and electron microscopic data for *Paramarteilia orchestiae*, the type species of that genus originally described from the amphipod *Orchestia*; *in situ* hybridisation shows that *Paramarteilia* also infects crab species. We present, to our knowledge, the first known results of a paramyxid-specific DNA survey of environmental (filtered water, sediment) and

organismally-derived samples, revealing new lineages and showing that paramyxids are associated with a wider range of hosts and habitat types than previously known. On the basis of our new phylogeny we propose phylogenetic hypothesis for evolution of life cycle and infectivity traits observed in different paramyxid genera.

Introduction

Paramyxida (Rhizaria, Ascetosporea) are related to haplosporidians, paradinids and mikrocytids (Bass et al., 2009; Hartikainen et al., 2014a,b), although the evolutionary relationships among the five ascetosporean orders are currently unresolved. Paramyxids are apparently exclusively parasites of marine invertebrates - annelids, crustaceans and molluscs. Five genera have been recognised: *Marteilia, Paramarteilia, Marteilioides, Paramyxa* and *Paramyxoides*. However, Feist et al. (2009) suggested that *Marteilioides* and *Paramyxoides* should be suppressed and that *Marteilioides chungmuensis* be reassigned to *Marteilia*, *Marteilioides branchialis* to *Paramarteilia* and *Paramyxoides* to *Paramyxa*. One of the aims of the present study was to assess this recommendation by applying the first molecular phylogenetic approach to the group as a whole.

Paramyxids are increasingly recognised as pathogens causing economically significant mortalities of bivalves. The best known of these are marteiliosis/Aber disease in the European flat oyster *Ostrea edulis* and QX disease in the Sydney rock oyster *Saccostrea glomerata*, caused by *Marteilia refringens* and *M. sydneyi*, respectively (Perkins & Wolf, 1976; Berthe et al., 2004); *M. refringens* is currently listed as notifiable to the World Organisation for Animal Health (OIE, 2018). *M. sydneyi* has previously been listed as a notifiable disease, but is not currently subject to international legislation (OIE, 2018). Other significant bivalve diseases are caused by *M. cochillia* in cockles (Carrasco et al., 2012, 2013), *M. chungmuensis* in *Crassostrea gigas* in Korea and Japan (Comps et

al., 1987; Itoh et al., 2003a), and *M. granula* in the clam *Ruditapes philippinarum* in Japan (Itoh et al., 2014).

Paramyxids in crustaceans include *Paramarteilia canceri*, which causes disease of the edible/brown crab *Cancer pagurus* (Feist et al., 2009), and *P. orchestiae* in amphipods, where it has been investigated in relation to modification of their sexual status (Ginsburger-Vogel, 1991; Short et al., 2012a, b). However, beyond these very few examples there are so far no other reports of paramyxids causing disease in crustaceans, although more recently copepods have been shown to be vectors in the lifecycle of *M. refringens* (Carrasco et al., 2007a, b; Arzul et al., 2014).

Polychaetes are similarly understudied as potential hosts of paramyxids. Adlard and Nolan (2015) recently demonstrated that *M. sydneyi* cycles through both the polychaete *Nephtys australiensis* and the oyster *S. glomerata*, providing another example of the complexity of at least some paramyxid lifecycles. Otherwise the only known annelid-infecting paramyxid is *Paramyxa*, of which the only described species, *P. paradoxa*, was first described in a polychaete larva from Banyuls-sur-Mer on the Mediterranean French coast by Chatton (1911). No similar organisms were reported until a paramyxid parasite of the polychaete *Nephtys caeca* was described by Larsson and Køie (2005) as *Paramyxoides nephtys*, distinguished from *P. paradoxa* on the basis of spore shape and cytology. However, Feist et al. (2009) considered that the characters used to distinguish these two genera were taxonomically invalid and transferred *Paramyxoides* to *Paramyxa*.

Paramyxids are also commonly referred to as paramyxeans. This class/order discrepancy deserves some explanation, to clarify the actual classification of the group and to ground its nomenclature in a robust phylogenetic context, which is an important aim of this study. Like many enigmatic micro-eukaryote groups, paramyxid taxonomy has been historically unstable, partly due to high levels of phenotypic conservation and convergence commonly seen in protists, particularly parasites (Boenigk et al., 2012; Hartikainen et al., 2014b; Neuhauser et al., 2014; Poulin & Randhawa, 2015). The presence of haplosporosome-like bodies provided early evidence that *Marteilia* and *Paramarteilia* were related to haplosporidians (Perkins, 1979), and ultrastructural characteristics supported a relationship between these genera and the first described genus eventually assigned to paramyxids, *Paramyxa* (Chatton, 1911; Desportes & Lom, 1981). *Marteilia* and *Paramarteilia* were described later, in the 1970s (Perkins, 1976; Perkins & Wolf, 1976; Desportes & Ginsburger-Vogel, 1977; Ginsburger-Vogel & Desportes, 1979), as detailed in Desportes & Perkins (1990) and Feist et al. (2009). All three genera are distinguished from haplosporidians by the production of variable numbers of daughter cells endogenously formed within a primary amoeboid stem cell, leaving to their characteristic 'cell within cell' development. This group has been treated as a class (Paramyxidea Levine, 1980), phylum (Paramyxea Desportes & Perkins, 1990), and most recently as the order Paramyxida in Bass et al. (2009), which is both the original and most stable taxonomy, concordant with both molecular and morphological analyses (Cavalier-Smith & Chao, 2003a,b; Bass et al., 2009; Feist et al., 2009).

Environmental DNA (eDNA) sequencing studies (i.e. generating and sequencing PCR amplicons or metagenetic fragments from DNA/RNA extracted from environmental samples to assess their biodiversity) are beginning to reveal high levels of diversity within groups of known parasites (Bass et al., 2009; Hartikainen et al., 2014a, b), providing powerful insights into parasite lifecycles, environmental reservoirs and transmission routes, and previously unknown parasitic lineages. These approaches are seen as increasingly important for disease monitoring and prediction, and policy issues, as described in Stentiford et al. (2014) and Bass et al. (2015). Paramyxid 18S rRNA genes are phylogenetically divergent and therefore usually missed in broadly-targeted 18S sequencing surveys (Bass et al., 2015). In such cases PCR primers designed specifically for the group under study can be very valuable (Hartikainen et al., 2014a, b). One aim of this study was to design and optimise such a primer set to better understand paramyxid diversity and phylogeny.

As well as generating new eDNA-based sequences as described above, we also analyse all available paramyxid 18S rDNA sequences, providing a comprehensive paramyxid phylogenetic tree, in order to rationalise paramyxid nomenclature and determine their evolutionary relationships. We show that *Marteilia, Paramarteilia* and *Marteilioides* form highly distinct and robustly supported phylogenetic clades, confirming their validity as separate genera, and that all three genera form a robustly supported clade that also includes *M. granula* (recently described by Itoh et al., 2014), and uncharacterised environmental sequences, confirming the monophyly of the order Paramyxida.

Materials & Methods

Sample collection

For invertebrates, 150 mussels *Mytilus edulis* were collected from the River Tamar estuary mouth near Cremyll Ferry, Devon, UK in June and July 2013. The June individuals were incubated in sterile artificial sea water (ASW; Culture Collection of Algae and Protozoa (CCAP) recipe) in sets of 10 individuals (clustered according to sampling proximity) for 1 h. Post-incubation, 50-100 ml of water were syringe-filtered through Whatman GF/F filters (GE Healthcare, USA) and filters subsequently fixed in 100% molecular-grade ethanol. A further 150 individuals of *M. edulis* and 222 *Ostrea edulis* were similarly collected from a nearby site, Jupiter Point (River Lynher, Tamar Estuary, UK), in September 2015. All bivalves were dissected and tissue cross-sections including digestive gland and mantle were fixed in Davidson's Solution for histology, glutaraldehyde for transmission electron microscopy (TEM), and 100% ethanol (June samples) or flash frozen in liquid nitrogen (July samples) for molecular analyses. Other invertebrates (polychaetes, amphipods, shrimp, barnacles) were also samples from sediments and under rocks in the mussel sampling areas. Animals were kept intact and preserved in 100% molecular ethanol at -20 °C until DNA extraction. Amphipods *Orchestia gammarellus* were collected at low tide in the intertidal zone above the high water mark at Castle Cove, Weymouth, England (50° 35' 45.6' N, 2° 27' 36' W; n=178) between September 2014 and February 2014 and in the Gann Estuary, Dale, Wales (n=197) during November 2014. For *O. gammarellus*, morphological identity was confirmed, length was measured using callipers, sex was determined and any external abnormalities, i.e. lost limbs or notable markings, were recorded. The *O.*

gammarellus were anaesthetised using clove oil (Eugenol 80-90%) at a dilution of 0.2 µl/ml of seawater and were transversely sectioned into three using a stereomicroscope (Leica M125, Leica, Germany). One section was placed in 100% ethanol for molecular work; the second section was placed in a 2.5% glutaraldehyde 0.2 M sodium cacodylate buffer for TEM and the final section was palced into a cassette in Davidson's Seawater Fixative for 24 h for histopathology and *in situ* hybridisation (ISH).

Edible crabs *Cancer pagurus* were captured in baited traps from the commercial fishery in Weymouth Bay in January 2004. A total of 30 crabs were transported back to the Cefas Weymouth laboratory, where they were anaesthetised on ice for 30 min before dissection. Hepatopancreas, heart, gill, muscle and gonad tissues were fixed in Davidson's sea water fixative for histology and hepatopancreas and gonad samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer for TEM.

Spider crabs *Maja squinado* were captured using a Granton trawl on board the Cefas Endeavour from the Cardigan Bay area, Wales, in July 2008. As for edible crabs, 30 spider crabs were anaesthetised on ice for 30 min before dissection; hepatopancreas, heart, gill, muscle and gonad tissues were fixed in Davidson's sea water fixative for histology and hepatopancreas samples were fixed in 2.5% glutaraldehyde in 0.1M sodium cacodylate buffer for TEM.

Polychaete worms (100 specimens, mostly *Nephtys caeca*) were collected from the tidal, brackish Fleet lagoon, Weymouth, Dorset, UK (salinity 10-30ppt) on 15

May 2015. Each worm was dissected into three sections in the field and fixed for molecular analyses, histology and TEM.

For environmental samples, 150L water samples were collected at three sites in the Tamar estuary: Cremyll Ferry, Wilcove and Neal's Point. Each was passed serially through 50 µm and 20 µm meshes. Material collected on the meshes (filtrand) was transferred to 2 ml cryotubes and fixed in 100% ethanol. A 50 L aliquot of water from each site was kept cool and in the dark and transported back to the laboratory within 24h, where aliquots were filtered under pressure onto 142 mm 0.45 µm cellulose acetate filters (Sartorius, Germany) and immediately stored at -80 °C. Littoral sediment samples (0.5-1 g), from the areas in which mussels were sampled, were taken from the Cremyll site and fixed in 100% ethanol. Water and sediment samples were collected using the same protocols from Newton's Cove and the Fleet lagoon in June and October 2011 and April 2012. Filtered freshwater and littoral marine water were similarly sampled (but without the 0.45 µm filtering step) and benthic sediments from sites in the Western Cape, South Africa (10x water samples, 14x sediment and sand samples), Sabah, Borneo, Malaysia in December 2011 (28x water samples) and various sites in Florida, USA in June 2014 (27x water samples, 34x invertebrate incubations (as for *M. edulis* incubations, above)). Water from shrimp hatchery tanks at the Borneo Marine Research Institute (University of Malaysia, Sabah) was sampled 5x also as for *M. edulis* incubations.

Sample processing and DNA extraction

Sediment and 50 µm and 20 µm fraction filtrand samples were freeze-dried at -40 °C until dry. DNA was extracted from these and the 0.45 µm filtrand using

the PowerSoil DNA Isolation Kit (MoBio Laboratories, USA). DNA was extracted from invertebrate (apart from amphipod) tissue from all sites using the DNEasy Blood and Tissue Kit (Qiagen, Germany). Flash frozen mussel tissue was defrosted into RNALater (Qiagen) before DNA extraction using the 96-well DNEasy Blood and Tissue Kit (Qiagen).

For the amphipods, the 100% ethanol-preserved samples were suspended in a solution of Lifton's buffer (2.3% w/v sucrose, 1 M Tris pH 8.0, SES, 0.5 M EDTA pH 8.0) containing proteinase K (10 mg/ml). Following incubation overnight at 55 °C, DNA was extracted using a phenol chloroform extraction method with ethanol precipitation (Nishiguchi et al., 2002). The resulting DNA was suspended in 40 µl of sterile water and the DNA concentrations of each sample were quantified by spectrophotometry (NanoDrop ND-1000) and the Quantifluor DS-DNA system (Promega, UK).

Filters from invertebrate incubation samples were freeze-dried at -40 °C for 2 h to remove ethanol. Dried filters were subsequently kept on ice and cut into small pieces using sterile scissors prior to DNA extraction using the DNEasy Blood and Tissue Kit (QIagen).

PCR and Sequencing

A nested primer set targeting regions V7 and V8 of the paramyxid 18S rRNA gene was designed, based on all available paramyxid sequence data in June 2013. The first round PCR used primers Para1+fN (GCG AGG GGT AAA ATC TGA T) and ParaGenrDB (GTG TAC AAA GGA CAG GGA CT). Second round PCR used primers Para3+fN (GGC TTC TGG GAG ATT ACG G) and Para2+rN

(TCG ATC CCR ACT GRG CC) (primer set A). All PCRs were conducted in 20 µl final volumes with 1 µl of template DNA and a final concentration of 0.5 µM of each primer, 0.4 mM dNTPs, 2.5 mM of $MgCl_2$, 1X Promega Green Buffer and 0.5 U of Promega GoTaq. Cycling conditions for first round PCR consisted of a 3 min denaturation at 94 °C, followed by 42 cycles of 95 °C for 30 s, 67 °C annealing for 1 min and 72°C for 1 min. Amplicons were extended by a final incubation at 72 °C for 5 min and stored at 4 °C. Second round PCR used 1 µl of first round product as template DNA, and the cycling conditions were altered to an annealing temperature of 62 °C. These primers were used to screen environmental and invertebrate tissue/incubation samples from Newton's Cove, Fleet, Tamar estuary, Florida and Borneo, except those detailed in the following paragraph.

Following the publication of sequence data for '*Marteilia granula*' (Itoh et al., 2014), primer set A was modified to include this sequence type (primer set B). The resulting hemi-nested PCR protocol used Para1fGW (GGG CGA GGG GTA AAA TCT) and ParaGENrGW (GTG TAC AAA GGR CAG GGA CT) (first round), followed by ParaGen3fGW (GGC TTY TGG GAG AKT ACG GC) and ParaGenrGW (second round). PCR mixtures were prepared as above. Cycling conditions consisted of a 5 min denaturation at 95 °C, followed by 30 cycles of 95 °C for 1 min, 50 °C annealing for 1 min and 72 °C extension for 1 min. Amplicons were extended by a final incubation at 72 °C for 10 min and stored at 4 °C. The same cycling conditions were used for both rounds of the hemi-nested PCR. These primers were used to screen the polychaete worms from the Fleet lagoon in May 2015, *O. edulis* and *M. edulis* tissues from Jupiter Point (Tamar, UK) collected in September 2015, and eDNA from South Africa. A

panel of samples comprising representatives from each sample set screened using primer set A was screened with primer set B to test for additional diversity not detected by primer set A. No differences between the diversity detected and frequency of paramyxid-positive PCRs were detected in these samples.

Fragments were visualised on 1.5% agarose gels stained with GelRed (Biotium, US). Amplicons were Sanger sequenced in one direction using primer Para3+fN or Para3fGW. Where direct sequencing produced a mixed product (Cremyll sediment, Wilcove water samples), amplicons were pooled from all PCR-positive samples and clone libraries were prepared using the StrataClone PCR cloning kit (Agilent Technologies, USA). Eight clones from each pool were sequenced in one direction using the M13R primer.

Phylogenetic analyses

All available paramyxid sequences were downloaded from National Centre for Biotechnology Information (NCBI, USA) GenBank including Blastn searches to identify uncharacterised (including environmental) sequences related to known taxa. These were aligned with sequences generated in this study using MAFFT version 7, e-ins-I algorithm (Katoh & Standley, 2013). The resulting alignment (47 sequences, including haplosporidian outgroup. 1812 positions analysed; amplicons generated using primer set A = 454-477bp; primer set B = 533-572bp) was refined manually and analysed using Maximum Likelihood (ML) in RaxML BlackBox version 8 (Stamatakis, 2014) (Generalised time-reversible (GTR) model with CAT approximation (all parameters estimated from the data); an average of 10,000 bootstrap values was mapped onto the tree with the highest likelihood value). A Bayesian consensus tree was constructed using

MrBayes v. 3.2.5 (Ronquist et al., 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for two million generations each with one cold and three heated chains. The evolutionary model applied included a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. all parameters were estimated from the data. Trees were sampled every 1000 generations and the first 500,000 generations were discarded as burn-in (trees sampled before the likelihood plots reached stationarity) and a consensus tree was constructed from the remaining sample. Sequences generated by this study are available from NCBI GenBank (Accession numbers KX259318-KX259327) and indicated on Fig. Ch1-01.

Histology and in-situ *hybridisation (ISH)*

Following 24 h fixation, samples were suspended in 70% industrial methylated spirits (IMS) before being dehydrated and infiltrated with paraffin wax using a vacuum infiltration processor (Peloris, Leica, UK). Wax embedded samples were trimmed along the sagittal plane using a rotary microtome (Shandon Finesse 325, ThermoFisher, UK) to expose tissue. Once trimmed, sections 3-4 µm thick were mounted onto glass slides and stained using H&E in an autostainer (Surgipath, UK) and then coverslipped (ClearVue, ThermoFisher, UK). Screening of samples for pathogens was performed using a Nikon Eclipse E800 light microscope (Nikon, UK). Digital images and measurements were captured using the integrated LEICA (Leica, UK) camera and LuciaG software (Nikon).

ISH was carried out on *Orchestia gammarellus* slides to localise *Paramarteilia orchestiae*, and *Cancer pagarus* and *Maja squinado* slides for *Paramarteilia* sp.

Probes were generated by PCR using *Paramarteilia*-specific primers Porchest298f (CTG ATG AGC CTG GCA AGA CCA C) and Porchest 396r (TGG GGC ACA CCG ATA CTG GG), producing a 98bp amplicon specific to the clade marked '*Paramarteilia'* on Fig. Ch1-01. The process was also carried out on *Nephtys caeca* slides for *Paramyxa nephtys*: *Paramyxa*-specific probes were generated using primers Paramyxa240f (AGC AGA CCA ATC GCT CGA) and Paramyxa449r (GAC TCA TTC GTG GCG CGT TT), producing a 209 bp amplicon. In each case probes were digoxigenin (DIG)-labelled using digoxigenin-11-dUTP in PCRs of 100 µl volume with a final concentration of 1x Promega colourless buffer, 2.5 mM $MgCl_2$, 20 µM PCR DIG labelling mix (Roche, Switzerland), 0.5 µM of each primer, 0.5 U of Promega GoTaq and 6 µl of template DNA. Amplifications were performed on a Peltier PTC-225 thermal cycler. Cycling conditions consisted of a 5 min denaturation at 94 °C, followed by 40 cycles of 95 °C for 30s, taxon-specific annealing temperature for 45 s (60 °C for *Paramarteilia*; 55 °C for *Paramyxa*), and 72 °C for 1 min. Amplicons were extended by a final incubation at 72 °C for 5 min and stored at 4 °C. Tissue sections 4 µm thick from histologically-positive individuals were mounted onto Poly-L lysine slides. These were deparaffinised, rehydrated and then treated with proteinase K solution (10mg/ml) for 30 min at 37 °C in a humid chamber. Proteolysis was terminated by incubating the slides in 100% IMS for 5 min and rinsing the slides with 2x SSC buffer for 5 min at room temperature. Sections were overlaid with a hybridisation solution (4x SSC buffer, 50% formamide, 1x Denhardt's solution, 10% dextran sulphate, 250 µg/ml yeast tRNA) containing the probe DNA (50:50 v/v). Slides were heated to 95 °C for 5 min and hybridised overnight at 42 °C. After hybridisation, sections were washed with 1X SSC buffer and 0.5X SSC buffer for 15 min at 42 °C. Slides were blocked with

6% non-fat milk in Tris buffer (pH 7.5) for 1 h at room temperature. The reactions were then developed with anti-DIG antibody conjugated with an alkaline phosphatase, nitroblue tetrazolium (NBT) and 5-bromo-4-chloro-3-indoylphosphatase (X-phos). The sections were counterstained with Nuclear Fast Red and examined under light microscopy. Negative controls lacked the DIG-labelled probe in the hybridisation buffer.

Transmission electron microscopy (TEM)

Selected parasite-positive animals were removed from glutaraldehyde and sectioned into 1mm^3 tissue blocks. The samples were fixed in 2.5% glutaraldehyde in 0.1 M sodium cacodylate buffer (pH 7.4) for 2 h and post-fixed by rinsing them in 1% osmium tetroxide in 0.1 M sodium cacodylate buffer (1 h). The samples received two rinses in 0.2 M sodium cacodylate buffer (10 min) before being dehydrated through a graded acetone series (10%, 30%, 50%, 70%, 90% and 100%) with 10 min in each solution. Samples were then infiltrated by Agar 100 epoxy resin (Agar Scientific, UK) and embedded by polymerising the samples at 60 °C overnight. Semi-thin sections (0.5 µm-2 µm) were taken from resulting blocks and stained with Toluidine Blue. Stained semi-thin sections were surveyed using a light microscope to identify target regions, and 70-90nm ultra-thin sections of these regions were mounted on uncoated copper grids. Finally, the samples were stained with 2% uranyl acetate solution followed by Reynolds' Lead Citrate (Reynolds, 1963) before being examined using a transmission electron microscope (JEOL JEM 1210, Japan). Digital images were obtained using Gatan Digital Micrograph software with a Gatan Erlangshen ES500 W camera.

Results

Paramyxid diversity

Table Ch1-T01 (at the end of this chapter) summarises all paramyxid genera and species for which 18S rDNA sequence data are available in public databases and/or are robustly identified morphologically (as a result of our literature survey), indicating their known host ranges and geographical distributions. The new data generated by this study are also included in the table.

Paramyxid 18S rDNA phylogeny

Bayesian and ML analyses of all currently known and newly generated paramyxid 18S rDNA data shows that the genera *Marteilia, Paramarteilia* and *Marteilioides*, and a newly sequenced parasite of *Nephtys caeca* and other polychates, group separately from each other, each in robustly supported clades of congeners and/or environmental sequences (Fig. Ch1-01A). *Eomarteilia* (previously *Marteilia*) *granula* does not branch with other *Marteilia* spp., but is sister to all other known paramyxeans with moderate to strong support in ML and Bayesian analyses with maximal taxon sampling (Fig. Ch1-01A). We therefore reassign this to the new genus *Eomarteilia*.

Although diversity within each of the genus clades is not high, some other relevant points arise from the phylogenetic analyses, as discussed below.

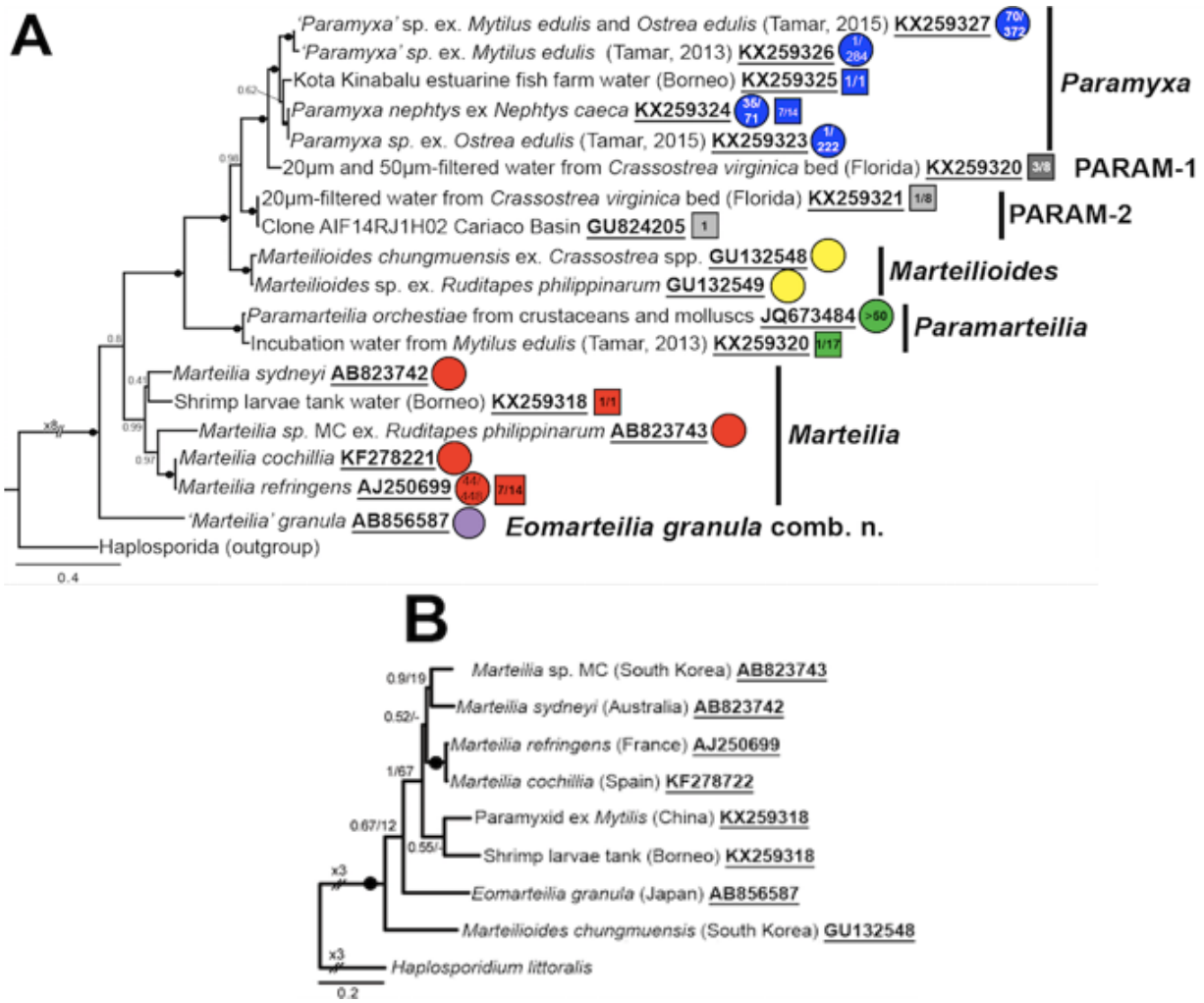

Figure Ch1-01. 18S rDNA phylogenies of paramyxids and relevant GenBank accession numbers. **A**. phylogeny of paramyxids generated using Bayesian Inference. Values on nodes indicate Bayesian Posterior Probabilities (PP); filled circles on nodes indicate maximal support (PP=1.0). Coloured squares indicate lineages detected in environmental samples, and circles those detected in host-associated (tissue or incubation) samples. Triangles indicate lineages for which only environmental sequences exist. Values in shapes indicate prevalence (number of positive samples/number screened). Where no value is present, the lineage was not detected in this study. **B.** More complete phylogeny of *Marteilia, Eomarteilia* and *Marteilioides* generated using Bayesian Inference, showing Maximum Likelihood bootstrap and Bayesian PP values.

Marteilioides

The *Marteilioides* clade has two distinct, known sister lineages, one (*M. chungmuensis*) from two *Crassostrea* spp. (*C. gigas* and *C. ariakensis* from Japan and South Korea; a total of five sequences in GenBank), and the other (undescribed *Marteilioides* sp.) from two independent studies in the Manila clam *Ruditapes philippinarum* (two GenBank sequences) (Yanin et al., 2013; first observed by Lee et al., 2001).

Marteilia

The *Marteilia* clade contains sequences which cluster in rough agreement with their geographical provenance: *M. refringens* and *M. cochillia*, sampled on many independent occasions from Europe and *M. octospora* from Spain (Ruiz et al., 2016) share very similar 18S sequences, and form a strongly supported clade with the highly distinct *M. sydneyi* sequence from *Saccostrea glomerata* from Queensland, Australia, '*Marteilia* sp. MC' from *Ruditapes philippinarum* in South Korea (Kang et al., unpublished data; sequence has GenBank accession AB823743), and another distinct sequence derived from a shrimp hatchery tank at the Borneo Marine Research Institute. As noted above, *M. granula* does not belong to this clade. A further sequence (not in GenBank) from *Mytilus* sp. from China was manually copied from Wang et al. (2012) and aligned with the *Marteilia* sequences in Fig. Ch1-01A. This is presented separately (Fig. Ch1-01B) as the 638bp fragment does not overlap with the Bornean shrimp sequence (with which it groups but with no support), but otherwise optimising the alignment between other *Marteilia* clade sequences. This tree does not differ significantly from the comparable part of Fig. Ch1-01A but does show that the Chinese *Mytilus*-derived sequence does not group with named *Marteilia* spp. This reduced taxon-sampled tree is also interesting in that, in the absence of other genera, *Eomarteilia* and *Marteilia* form a clade. After our analyses had been performed, *Marteilia octospora* was described by Ruiz et al (2016). The short 18S fragment available for *M. octospora* (within KU641125), although not in the most variable region of the gene, is almost identical to the corresponding region of *M. cochillia* (Fig. Ch1-01B).

Paramarteilia clade

Sequences in the *Paramarteilia* clade were recovered from mussel-, amphipod- and crab-associated material, and comprise two distinct but closely related sequence types: one only from crustaceans to date (amphipods from the genera *Echinogammarus (*Short et al., 2012a, b, 2014) and *Orchestia* (this study)), and in incubation water from *C. pagurus* and *Cerastoderma edule*. The other 18S sequence type has to date only been detected in *M. edulis* incubation water. ISH probes designed for the two *Paramarteilia*-infected tissue in crabs *Cancer pagurus* (connective tissue within hepatopancreas, heart, ovary, testicular follicles) and *Maja squinado* (hepatopancreas). The histology and TEM of *Paramarteilia* in *C. pagurus* and *M. squinado* are reported below.

Paramyxa clade

A paramyxid found in the polychaete *Nephtys caeca* in this study (assigned to *Paramyxa nephtys* as described below and labelled as such in Fig. Ch1-01A) shares an identical sequence to 0.45 µm-filtered water from Wilcove on the Tamar estuary (not separately shown on Fig. Ch1-01A). Related but not clearly distinct sequences were detected in a single *Ostrea edulis* sample from the Tamar estuary, UK, and eDNA from an estuarine fish farm in Borneo. A further two sequences, labelled '*Paramyxa'* to tentatively assign them to this genus pending ongoing morphological analysis, were detected in DNA extracted from bivalve digestive gland (DG) samples from the Tamar, UK. One of these was detected at a relatively high frequency in *O. edulis* (62/222; 28%) and *M. edulis* (9/150; 6%), but only in samples taken from Jupiter Point (Tamar) in September

2015. The other sequence type was detected only in 1/284 *M. edulis* DG from the 2013 Tamar sampling.

Paramyxid parasite of N. caeca *and other polychaetes*

Histological analysis showed that 23/71 *Nephtys caeca* specimens sampled from the Fleet lagoon (Weymouth, UK) in May 2015 were infected with an unknown paramyxid (Fig. Ch1-02). Three of these exhibited heavy infections of an ellipsoid spore-forming parasite typically restricted to the intestinal tract of the worm, including the mouth, the intestinal epithelium and lumen along the full length of the worm. TEM analyses of these heavily infected individuals revealed spore sacs with striated projections and containing four spores, very similar to those shown for *P. nephtys* in Larsson and Køie (2005). Also concordant with the description of *P. nephtys*, the developmental stages of the parasite had penetrated the intestinal epithelium and replicated to replace a large proportion of the host tissue. Mature stages were released from the intestinal cells into the lumen. No host response to infection was noted in the epithelium or lumen. Pre-spore stages were also similar to those described for *P. nephtys*. When all 71 *N. caeca* samples were screened using paramyxid-specific primers a further 13 (i.e. a total of 36 *Nephtys* individuals) were PCR-positive. Small samples of other polychaete species were collected from the same site as the *N. caeca* specimens. DNA from tissue of some of these was also paramyxid PCR-positive and yielded the same 18S sequence type in 3/5 *Nereis* sp. individuals, 2/14 *Nemertea*-like worms, 1/1 *Ophelia*-like worm and three unidentified polychaete individuals. We refer to this parasite as *Paramyxa nephtys* rather than *Paramyxoides* for reasons discussed further in this report.

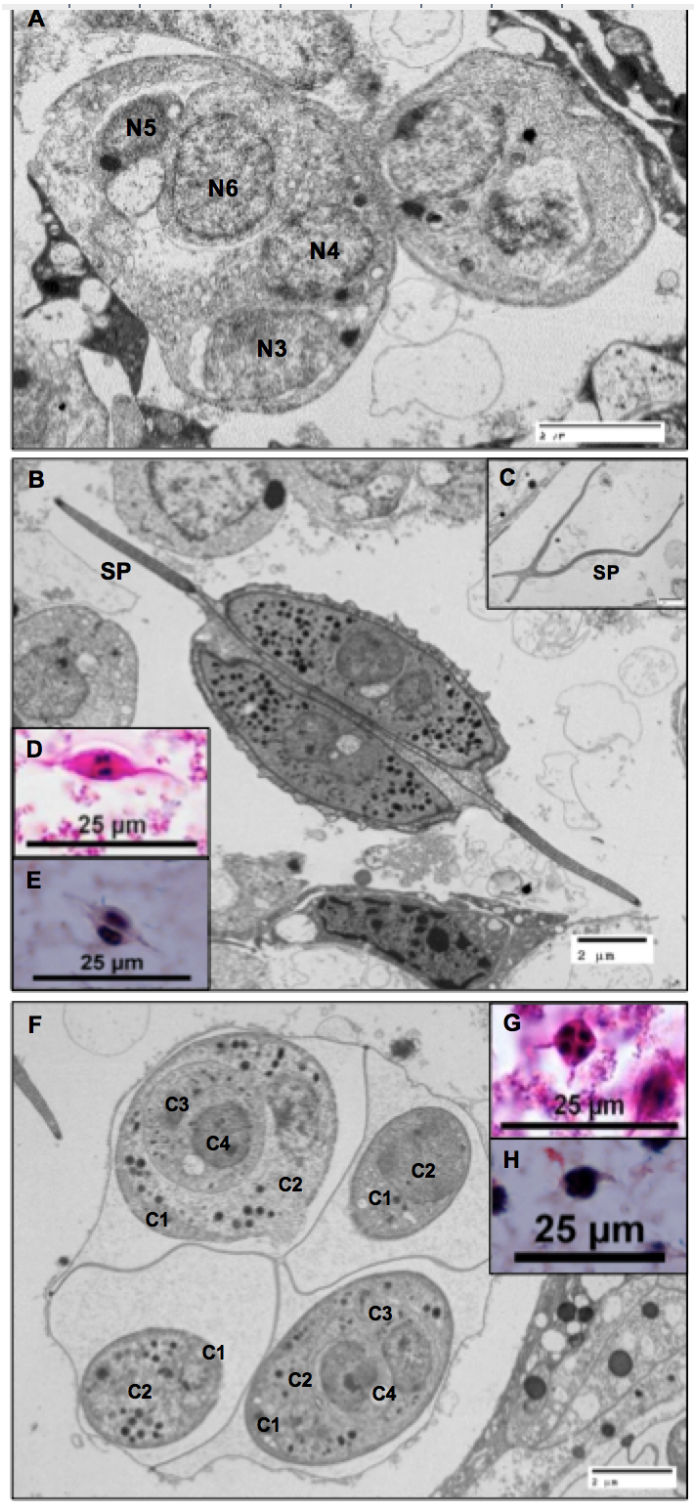

Figure Ch1-02. Transmission electron micrographs and supporting light micrographs (H&E), *in situ* hybridisation of developing and fully matured morphological *Paramyxa nephtys* cells. **A.** Developing *P. nephtys* cells detailing the third to sixth nuclei (N3-N6) of a developing *P. nephtys* spore. Transmission electron micrograph. Scale bar= 2 µm. **B.** Longitudinal view of two mature *P. nephtys* spores encased within spore sacs possessing striated projections. Transmission electron micrograph. Scale bar = 2 µm. **C.** Top-down view of *P. nephtys* spore sac terminal striated projection showing the projections form a single structure. Transmission electron micrograph. Scale bar= 2 µm. **D.** Longitudinal view of two mature *P. nephtys* spores. H&E stain. Scale bar= 25 µm. **E.** Longitudinal view of two mature *P. nephtys* spores. *In situ* hybridisation. Scale bar= 25 µm. **F.** Transverse section of four coupled *P. nephtys* spores demonstrating the four tetracellular spore arrangement (C1-C4). Transmission electron micrograph. Scale bar= 2 µm **G.** Transverse section of *P. nephtys* spores. H&E stain. Scale bar= 25 µm. **H.** Transverse section of *P. nephtys* spores. *In situ* hybridisation. Scale bar= 25 µm.

Paramyxid-specific eDNA analysis

Two lineages in Fig. Ch1-01A, marked as PARAM-1 and -2, have no characterised members, i.e. they have been detected only in eDNA samples. PARAM-1 comprises three identical sequences from marine sites in Florida, USA: one from a littoral filtered water sample and two from filtered water sampled within a *Crassostrea virginica* bed at Seahorse Key. In PARAM-2, two identical sequences (represented by GU824205 on Fig. Ch1-01A) were sequenced from the same sample (and possibly the same organism) in a eukaryote-wide survey of filtered water from the Cariaco bay, Venezuela (Edgcomb et al., 2011). The other very closely related sequence in PARAM-2 was amplified by our paramyxean-specific PCR protocol from 20 µm-filtered water from a bed of *C. virginica* adjacent to the Whitney Laboratory for Marine Bioscience, Florida, USA. We cannot assume that PARAM-1 or -2 belong to the genus *Paramyxa* as we have no morphological data for them.

Some of the other paramyxid clades were also represented in our eDNA screening, as shown in Fig. Ch1-03. *Marteilia refringens* 18S rDNA was amplified from sediment and filtered water column samples from Wilcove and Cremyll in the Tamar estuary (but not Neal's Point, furthest from the sea), where it was also detected in *M. edulis* tissue samples (5/144 mantle; 37/287 DG) and filtered incubation water (2/17 samples). However, no sequences corresponding to *M. sydneyi, Eomarteilia granula* or either *Marteilioides* sequence type were recovered from the eDNA screens.

The *Paramarteilia orchestiae* 18S sequence type was detected most frequently in amphipod tissue samples (whole animals) but the same sequence was also

detected in *Cancer pagurus* incubation water. A closely related sequence (98% similarity) was recovered from *Mytilus edulis* incubation water (1/17 samples). In the *Paramyxa* clade, the only PCR amplifications from 'environmental' samples were of the *P. nephtys* 18S-type in *Mytilus* incubation water, and the *P. nephtys*-like sequence type from the Borneo fish farm.

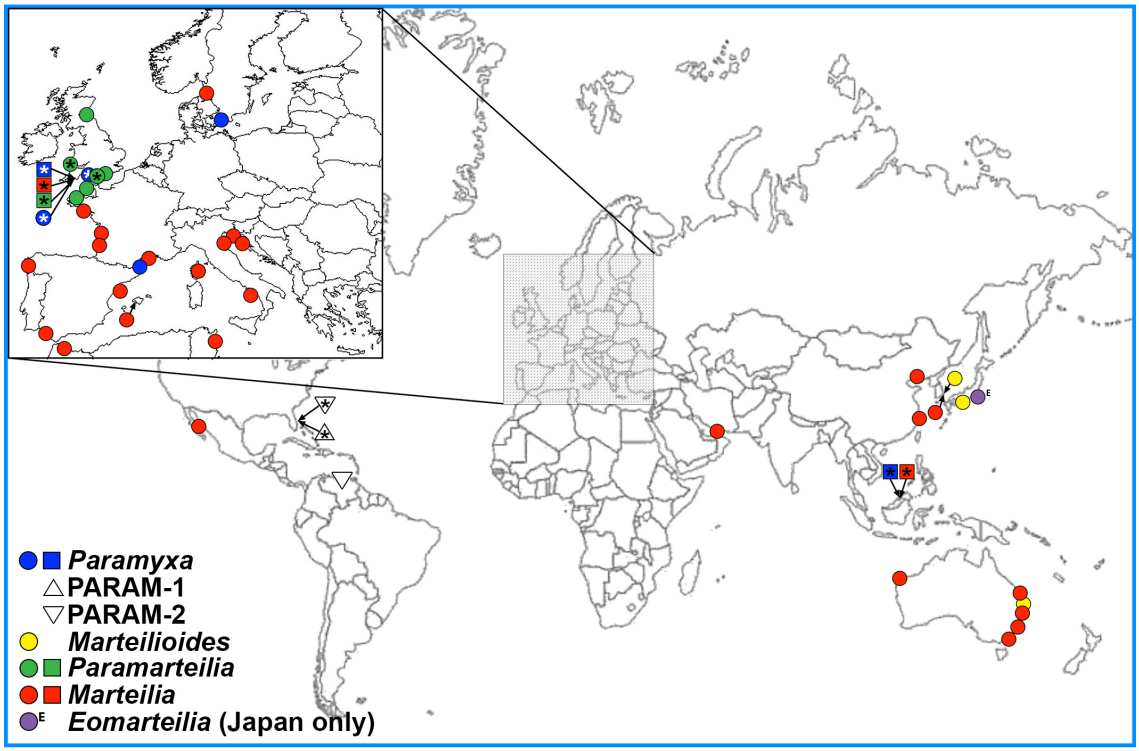

Figure Ch1-03 World map showing distribution of paramyxid clades, including only reports confirmed by sequence data or unambiguous microscopy. Coloured squares indicate detection of a lineage in environmental samples, and circles those detected in confirmed hosts or host-associated samples. Triangles indicate lineages for which only environmental sequences exist. Shapes labelled with asterisks represent lineages detected in this study. The inset shows the distribution of paramyxids within Europe.

Geographical distribution of paramyxids

Fig. Ch1-03 suggests strong biogeographical structuring of paramyxid diversity, and that this to an extent reflects the phylogenetic clustering. *Paramarteilia* and *Paramyxa* spp. are known only from Europe, *Eomarteilia* from Japan and *Marteilioides* from the Far East (South Korea, Japan, eastern Australia). The *Marteilia* clade is apparently more widely distributed: *M. refringens, M. cochillia* and *M. octospora* mostly from Europe (other than one record from the Pacific

coast of Mexico (Grijalva-Chon et al., 2015) and another from Kuwait), *M. sydneyi* from Australia, and many additional '*Marteilia* sp.' reports unconfirmed by sequencing in the literature therefore not included in Table Ch1-T01 or Fig. Ch1-03. The *Marteilia* clade is also represented in Borneo by an environmental sequence.

The environmental clades PARAM-1 and -2 were also only detected in a small number of samples: PARAM-1 in multiple samples from a single site in Florida, USA, and PARAM-2 from low latitude American continent sites (Florida and Venezuela), despite the fact that paramyxid-specific PCR was carried out on eDNA samples from Europe, the Americas, South Africa and Borneo. None of the South African eDNA samples were paramyxid-positive.

Paramarteilia: confirmation of type species and infections in crab spp.

We present the first known 18S rDNA sequence for the *Paramarteilia* type species *P. orchestiae* from the type species host *Orchestia gammarellus*, with histopathology and TEM analyses of the corresponding material (confirmed by *Paramarteilia*-specific ISH; Fig. Ch1-04C). Our light and ultrastructural observations were entirely concordant with the original description of *P. orchestiae* (Ginsburger-Vogel & Desportes, 1979). The parasite's primary cells were between 5 and 12 µm in diameter and contained multivesicular bodies with spherical vacuoles and electron dense, cylindrical bacilliform haplosporosomes. Up to nine secondary cells (sporonts, C2) were observed, each individually between 3 and 7 µm in diameter, and unlike the primary cells lacking haplosporosomes and with increased ribosome density. Within the tertiary cell, two spores were present. Developmental stages of the parasite

were dispersed throughout *O. gammarellus* tissues and organs including the epidermal tissue (Fig. Ch1-04C, D), the connective tissue, heart and ganglia of the nerve cord (Fig. Ch1-04A). Furthermore, the cells apparently 'migrate' between organs and were present in the oocytes of two females (Fig. Ch1-04B), which supports the original trans-ovarial transmission hypothesis (Ginsburger-Vogel & Desportes, 1979). Although the bi- or tri-cellular stages of the spore were not observed, the host species, sites of infection and morphology of the parasite unambiguously confirm this parasite as *Paramarteilia orchestiae*. A total of 369 *O. gammarellus* individuals were screened by PCR using the *Paramarteilia* primers of Short et al (2012a), including those analysed for histology; 24 of these were positive (10.81%); eight from Weymouth and 16 from Dale. No obvious pathology was displayed in 15 of these 24 samples.

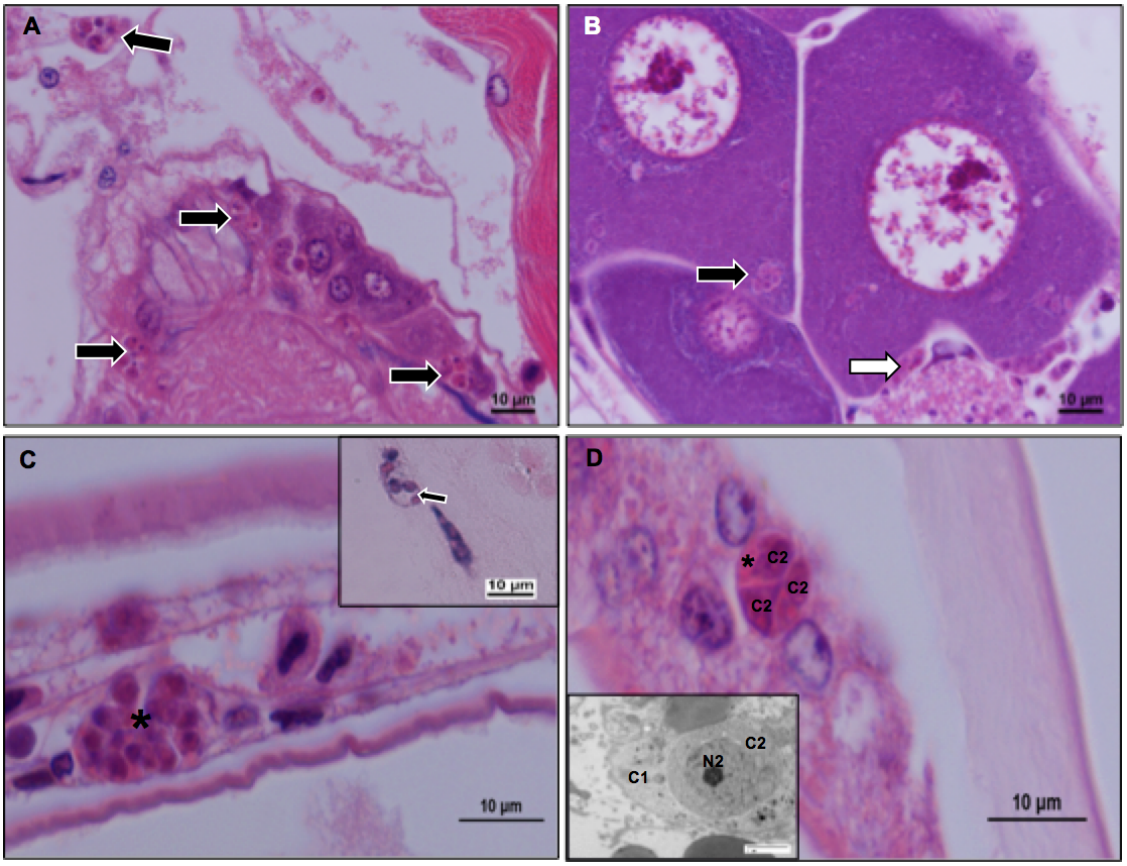

Figure Ch1-04. Light and electron micrographs depicting *Paramarteilia orchestiae* cells in *Orchestia gammarellus* tissue. **A.** Multiple groups of *P. orchestiae* cells (arrows) within connective tissues surrounding the ganglion of the nerve cord. H&E. All light micrograph scale bars= 10 µm. **B.** Intracellular *P. orchestiae* in oocytes (black arrow) and the connective tissue capsule surrounding the oocytes (white arrow). H&E. **C.** Cluster of *P. orchestiae* cells at different stages of development (asterisk) within the connective tissue of the leg. H&E. Inset: *in situ* hybridisation labelling of the *P. orchestiae* cells within the connective tissue of the leg (arrow). **D.** Individual *P. orchestiae* cell showing the cell-within-cell arrangement of the parasite. The primary cell (asterisk) contains secondary cells (C2). H&E. Inset: transmission electron micrograph of *P. orchestiae* cell demonstrating primary cell (C1) and a secondary cell (C2) containing nucleus (N2). Scale bar= 2 µm.

We also present the first known histopathology, ISH and TEM images of *P. canceri* in edible crabs and *Paramarteilia* in spider crabs *Cancer pagurus* and *Maja squinado*, respectively (Fig. Ch1-05). The morphology and infection characteristics of *Paramarteilia* in *C. pagurus* were consistent with those described for *P. canceri* in Feist et al. (2009) and the *Paramarteilia* infection in *M. squinado* was also very similar structurally. *Paramarteilia canceri* was observed in one of the 30 edible crabs sampled and *Paramarteilia* sp. was observed in two out of the 30 spider crabs sampled. developmental stages of

the parasite were dispersed throughout the connective tissues, hepatopancreas and gonad. The parasite is shown to infect the connective tissues surrounding the oocytes and the oocytes themselves, as well as the testicular follicles. The parasite in both crabs was similar to that observed in *O. gammarellus*, and that recorded from *Echinogammarus marinus* by Short et al. (2012b), the sequence of which is shown in Fig. Ch1-01A (JQ673484). However, more advanced developmental stages present in the crab species were not observed in amphipods. At present, based on morphological grounds it is not possible to propose that the same species infects these hosts. Because no 18S sequence for *P. canceri* exists (though the *P. orchestiae* 18S sequence was also detected in *C. pagurus* incubation water, and may correspond to *Paramarteilia* infecting edible crabs), and the known sequence variation with the *Paramarteilia* clade is very low (Fig. Ch1-01), we used the same ISH probe for *Paramarteilia* in both crab species (Fig. Ch1-05 insets).

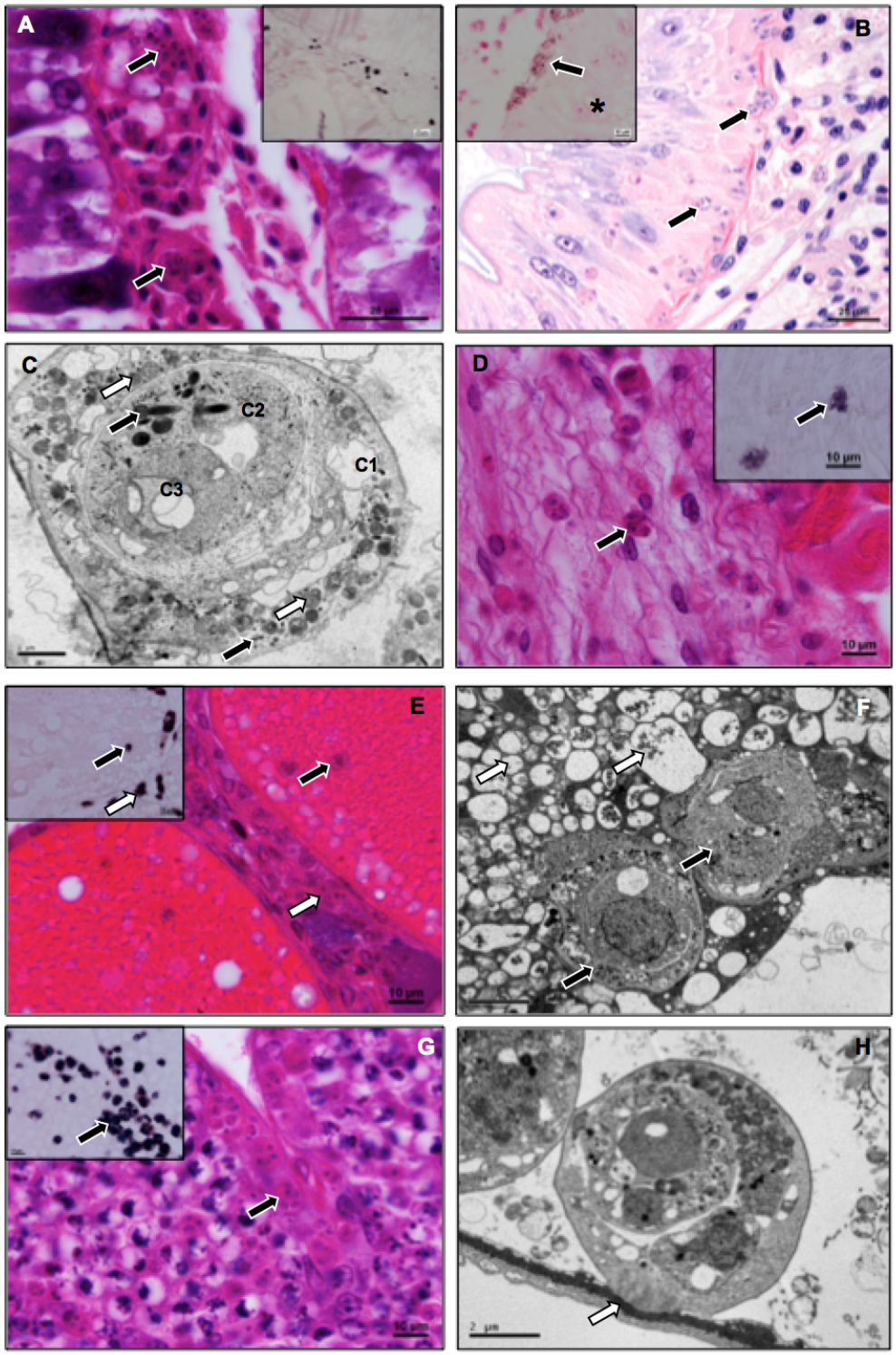

Figure Ch1-05. *Paramarteilia canceri* infecting edible crab *Cancer pagurus* tissues and *Paramarteilia* sp. infecting spider crab *Maja squinado* tissues. **A.** *P. canceri* infecting the connective tissue cells (arrows) within haemal spaces of the hepatopancreas. Inset: *in situ* hybridisation (ISH) labelling the *P. canceri* cells within the connective tissues in the hepatopancreas. Edible crab. H&E. Scale bar= 25 µm. **B.** *Paramarteilia* sp. within the hepatopancreatic tubule epithelial cells (arrows). Spider crab. H&E. Scale bar= 25 µm. Inset: ISH labelling of *Paramarteilia* sp. (arrow) at the base of the hepatopancreatic tubule epithelium (*). Spider crab. Scale bar= 10 µm. **C.** Transmission electron micrograph of *P. canceri* infecting the connective tissue cells within the heart detailing the cell-within-cell arrangement of the parasite. Primary cell (C1) contains secondary (C2) and tertiary (C3) cells, electron-dense haplosporosomes (black arrows) and multivesicular bodies (white arrows). Edible crab. Scale bar= 1µm. **D.** *Paramarteilia canceri* infecting the connective tissue cells (arrow) within the heart. H&E. Inset: ISH labelling of *P. canceri* infecting the connective tissue cells (arrow) within the

heart. Edible crab. Scale bar= 10 µm. **E.** Intracellular *P. canceri* infecting the ovary (black arrow) and the connective tissues of the ovary (white arrow). H&E. Inset: ISH labelling of *P. canceri* infecting the oocytes themselves (black arrow) and the connective tissues of the ovary (white arrow). Edible crab. Scale bar 10 µm. **F.** Transmission electron micrograph of *P. canceri* within a vitellogenic oocyte. The oocyte is almost completely filled with yolk globules (white arrow). Multicellular *P. canceri* parasites (black arrow) are clearly visible within the oocyte. Edible crab. Scale bar= 2 µm. **G.** *P. canceri* infecting the testicular follicles (arrow). H&E. Inset: ISH labelling of *P. canceri* infecting the testicular follicles. Edible crab. **H.** TEM of *P. canceri* within the testicular follicles. Parasite appears to be attached to the epithelium of the testicular follicle (arrow). Edible crab. Scale bar= 2 µm.

Discussion

To our knowledge the phylogenetic analysis presented here (Fig. Ch1-01A) is the first to show the relative branching positions of all paramyxid genera and species for which sequence data are available. The laterally compressed appearance of the tree may misleadingly imply low 18S sequence differences. In fact all the terminal branches represent distinct sequence types, with the possible exceptions of the two bivalve-derived *Paramyxa* sp. sequences at the top of Fig. Ch1-01A, and the Venezuelan and Floridean 18S-types in PARAM-2. For example, the clearly distinct *Marteilia cochillia* and *M. refringens* have 99% similar 18S sequences (1733/1742 identical nucleotide positions) and are very obviously different species based on phenotype. Many protistan species are identical, or nearly so, at the 18S level (Bass et al., 2009; Boenigk et al., 2012), yet show very different host associations and sporulation characteristics. Internal transcribed spacer (ITS) and intergenic spacer (IGS) regions are known to be more phylogenetically informative for the study of paramyxids, as shown by studies of *Marteilia* spp. by Kerr et al. (2018), and the determination of distinct ITS1 and ITS2 sequence types found in *Paramarteilia* spp. infecting amphipods *Orchestia gammarellus* and velvet crabs *Necora puber* (Ward et al., in prep). However targeting the relatively conserved 18S gene allows for the

development of more inclusive PCR primers targeting all known diversity within the group, and as demonstrated in this study is suitable for detecting novel sequence types both within known clades (*Marteilia, Paramarteilia*, PARAM-2) and previously unsequenced lineages.

It should however be noted that the ampicons generated in this study are relatively short, especially when compared to the whole 18S gene (450-572bp of 1812 positions analysed). There is little or no overlap with some existing sequence types, as demonstrated by the need for a second phylogeny in Fig. Ch1-01. Kerr et al. (2018) demonstrated that phylogenies including the full ribosomal array are more appropriate for resolving relationships between *Marteilia* spp. showing high identity in both 18S and ITS regions, and so future studies on paramyxid phylogeny should look to include a larger number of gene regions, and idealy move towards the generation of genomic and transcriptomic datasets to identify suitable markers for species delineation.

Some phylogenetic distinctions in Fig. Ch1-01A may reflect different host affiliations, for example the *Marteilioides chungmuensis* lineage is to date exclusively associated with the clam *Ruditapes philippinarum* and the distinct *Marteilioides* sp. with *Crassostrea* spp. The two closely related *Paramarteilia* 18S types may also have different host associations (molluscs versus crustaceans), although the ‘crustacean’ sequence has also been detected in *Cerastoderma* incubation water (but may not correspond to a parasite of the cockle), and the *Mytilus*-associated 18S type has to date only been detected once, also in (*Mytilus*) incubation water.

The only morphological characteristics thought to be useful to distinguish between paramyxid genera are the numbers of tertiary cells (C3, which then becomes the spore) produced and the number of cells constituting the spores (Feist et al., 2009). However we show here that these are not taxonomically reliable; the unrelated *Marteilioides* and *Marteilia* both form tri-cellular spores, although from different numbers of tertiary precursor cells- two in *M. sydneyi*, four in *M. refringens*, six in *M. cochillia* and eight in *M. octospora*. The only *Marteilioides* sp. for which the number of tertiary cells is known (*M. chungmuensis*) has a single tertiary precursor cell; the possibility that more tertiary cells occur in other *Marteilioides* lineages is too great for this character to be used to distinguish them from one another or from *Marteilia*. Furthermore, *Paramarteilia*, grouping between *Marteilia* and *Marteilioides* (Fig. Ch1-01A) forms bi-cellular spores while *Paramyxa* has tetra-cellular spores. Therefore there is no systematic variation in either tertiary cell number or numbers of cells constituting spores with the phylogenetic branching order.

We provide the first known molecular evidence for the phylogenetic position of *Paramyxa*/*Paramyxoides*. The parasite of *Nephtys caeca* that we analysed was ultrastructurally inseparable from the description of *Paramyxoides nephtys* by Larsson & Køie (2005). However, as the only consistent difference between *P. nephtys* and the earlier description of *P. paradoxa* (Chatton, 1911) is the presence of striated radiations on the mature spore tetrads, we agree with Feist et al. (2009) that *Paramyxoides* is a junior synonym of *Paramyxa* and now recognise the two species within the latter (original) genus: *P. nephtys* (this study) and *P. paradoxa*, which remains to be sequenced, but we suggest it is

likely to group with the '*Paramyxa*' sequences from bivalves or clades PARAM1/2.

In the absence of morphological data we refrain from assigning PARAM-1 or -2 to *Paramyxa*, the characterised genus to which these lineages are most closely and strongly related. PARAM-1 may turn out to be *P. paradoxa*; however the relationship between genotypic and phenotypic distance is not straightfoward, and decisions about taxonomic boundaries should be made taking into account both kinds of evidence (Boenigk et al., 2012). A good illustration of this is that three morphologically different *Marteilia* spp., *M. refringens*, *M. cochillia* and *M. octospora*, are extremely similar at the 18S level, yet *Marteilia* has been considered morphologically indistinguishable from *Marteilioides* and *Eomarteilia* spp., all three genera being very different from each other in terms of 18S sequence similarity. *Marteilia* and the morphologically similar but even more basally branching *Eomarteilia* may reflect the ancestral state for the whole order.

Paramyxa and *Marteilioides* form a robustly supported clade with PARAM-1 and -2, separated by maximal bootstrap support from other paramyxid genera. Therefore the suppression of *Marteilioides* as recommended by Feist et al. (2009) and assumed by Carrasco et al (2015) is invalid. However, *Marteilioides* remains a poorly sampled genus with only one described species. Sequence data are required to confirm whether '*Marteilioides*' *branchialis* groups with *M. chungmuensis* or in the *Paramarteilia* clade, as suggested by Feist et al (2009). *Paramarteilia* itself is sister to *Paramyxa*+*Marteilioides* with robust support, and is therefore not directly related to *Marteilia.*

We provide molecular (ISH) confirmation of *Paramarteilia* infection of edible and spider crabs. The description of *P. canceri* by Feist et al. (2009) is concordant with the parasite cells observed in both edible and spider crabs in this study (two bi-cellular spores). Our ISH results confirm that the parasite infecting both crab species belongs to *Paramarteilia*, and may be the same species of *Paramarteilia*. However, material was not available to generate an 18S sequence for *P. canceri* or the *Paramarteilia* from *Maja squinado*, so although the parasite of the latter was morphologically indistinguishable from that in *Cancer pagurus* we cannot yet confirm it is *P. canceri* until sequence data are available. Sequence data are required from both crab-infecting parasites also to confirm whether their sequence is identical to *P. orchestiae*. The role of paramyxids in crab disease has received almost no attention, and will almost certainly reward future disease investment.

Itoh et al. (2014) and Carrasco et al. (2015) tacitly concur with the Feist et al. (2009) report of suppression of *Marteilioides*, and include '*M*'. *granula* within the genus *Marteilia*. In the Neighbour-Joining (NJ) phylogeny of Itoh et al. (2014), '*M*'. *granula* is separated from other *Marteilia* spp. by *Marteilioides*, and in an ML analysis of the same taxon sample in the same paper and Carrasco et al. (2015), *'M'. granula* forms a weakly supported clade with other *Marteilia* spp. However, our phylogenetic analyses, which additionally include *Paramyxa* (sister to *Marteilioides*) and *Paramarteilia*, show that including *'M'. granula* within *Marteilia* would produce a paraphyletic *Marteilia*, as *'M'. granula* branches separately with strong support from the clade comprising *M. refringens, M. cochillia* and *M. sydneyi* in Fig. Ch1-01A. Therefore we suggest that *'M'. granula*

should be assigned to a new genus (*Eomarteilia*). Similarly, *Marteilioides* cannot be considered congeneric with *Marteilia* (See Feist et al., 2009; Carrasco et al., 2015) without incurring paraphyly of *Marteilia* and requiring that all other paramyxid genera be subsumed into *Marteilia*. It would be clearly undesirable and nonsensical to represent such a biological diversity of paramyxids as that illustrated in this study by a single genus.

It is worth noting that if an incomplete sampling of paramyxid diversity is used for phylogenetic analyses, the illusion may be given that *Eomarteilia* and *Marteilia* form a holophyletic clade (Fig. Ch1-01B), however this has very weak support compared with the more complete taxon sampling in Fig. Ch1-01A, emphasising the general desirability of comprehensive taxon sampling as a basis for the best possible phylogenetic interpretation. As addressed above, all of these phylogenetic relationships should be tested further by including more genes in phylogenetic analyses, when available.

Using paramyxid group-specific primers to screen environmental and organismal DNA samples for 'hidden' diversity is a powerful technique, revealing novel lineages and suggesting new ecological/host associations for verification (Moreira & Lopez-Garcia, 2002; Bass et al., 2015). We detected a *Paramarteilia* sequence type in *Orchestia gammarellus*, the histopathology and TEM of which was entirely concordant with the description of *Paramarteilia orchestiae* (Ginsburger-Vogel & Desportes, 1979). We then used ISH to confirm the presence of this sequence type in both *C. pagurus* and *M. squinado*. This approach can be used to determine whether, for example, our detection of *Paramyxa* sp. in *Mytilus* tissue represents actual infection or is more likely

trophic passage or accumulated by filtration, and to investigate different lifecycle stages and alternative hosts (Bass et al., 2015).

Some aspects of our eDNA results for different paramyxid lineages may be explained by life-history traits, for example sites of infection and modes of transmission. *P. orchestiae* and *Marteilioides* spp. are vertically transmitted (via host eggs); neither has been detected by eDNA methods in environmental samples. On the other hand, *Paramyxa nephtys*, *Marteilia refringens, M. sydneyi, M. cochillia* and *Eomarteilia granula* infect host digestive gland/gut tissue and are likely or known to be released from these tissues into the environment. The first two taxa in this list have been detected in environmental as well as organismal samples, and *M. refringens* has been shown to infect planktonic crustacean hosts. The low current sampling levels preclude any generalisations being made from these observations, but future results and experimental design should take them into account. It should also be acknowledged that techniques used for DNA extraction may bias results from environmental samples due to the inadequate lysis of paramyxid life-stages, or the presence of PCR inhibitors in extracted DNA. It is therefore important that the absence of a sequence type in a particular sample type should not be taken as definitive proof of its absence.

Furthermore, given the limited number and diversity of paramyxid sequence types available for use in primer design, it should also be considered that they require further revision in order to capture the full extent of 18S sequence variation within the order, and may be biased against more divergent lineages.

eDNA methods are also very useful for detecting the true geographical range of lineages, which as noted above is of particular interest in paramyxid studies. However, negative eDNA results are not conclusive, and the fact that we did not detect *M. cochillia, M. sydneyi, E. granula* or either *Marteilioides* 18S sequence types in our screens from the UK, Borneo, South Africa or Florida does not signify that these taxa are not more widely distributed than implied in Fig. Ch1-03. To address this, further probing of organismal and environmental samples from more areas is required, ideally using even more tightly lineage-specific primers than were used in this study to maximise detection sensitivity and specificity. In general, the group-specific eDNA results from paramyxids contrasts with that earlier obtained for Haplosporida (Hartikainen et al., 2014a), which revealed higher levels of diversity and higher detection frequencies from environmental samples. The paramyxid results are perhaps more akin to those for Mikrocytida (Hartikainen et al., 2014b), which were more limited in environmental samples and detection more strongly associated with potential hosts or particular environmental compartments. It may be that paramyxids and mikrocytids are more tightly host-associated than haplosporids and/or they less frequently infect small planktonic animals (and are consequently less likely to be captured by sampling of planktonic habitats) either as primary or alternative hosts.

A further complexity in the use of eDNA and host screening methods for the detection of novel and existing paramyxid diversity is a lack of understanding surrounding the role played by environmental conditions in the prevalence and pathogenicity of most lineages. Microscopy-based studies suggest the minimum water temperature of 18-20 °C necessary for gonad maturation in *Crassostrea*

gigas is similar to that required for development of *Marteilioides chungmuensis* within host oocytes (Kang et al., 2000; Ngo et al., 2003), and follow-up studies utilising both microscopy and PCR seem to confirm this (Tun et al., 2008). Temperature has also been shown to be a key parameter in the life cycle of *M. refringens* (Berthe et al., 1998) and *M. sydneyi* (Rubio et al., 2013). Therefore frequent collection of both organismal and environmental samples, paired with recording of environmental conditions, is essential to accurately determine seasonality and the factors which influence disease onset. The 'window of infection' (i.e. the timespan over which infection is observable in the primary host) also varies between taxa, and within the same taxa across several years. Onset of the infection window of *M. sydneyi* in *Saccostrea glomerata* often follows a rapid decrease in water salinity, and can last between 8 and 18 weeks, though the exact environmental conditions determining this window remain unknown (Rubio et al., 2013).

Our attempt to collate distribution and host association data from all recorded paramyxid species to date has, in tandem with the phylogenetic analysis, suggested a strong biogeographical structuring of paramyxid lineages. The closely related *M. refringens, M. cochillia* and *M. octospora* have only been recorded in Europe (the latter two only from Spain to date), whereas the more distantly related *M. sydneyi* has been reported only from Australia (both east and west coasts when non-sequenced records are considered), suggesting that geographically distant lineages are likely to be more distantly related. Further highly distinct lineages were sampled in Malaysian Borneo (from shrimp larvae tank water in a hatchery), South Korea (*Marteilia* sp. MC), and the Yellow Sea and East Sea coasts of China ('paramyxid ex. *Mytilus edulis'*), where both the

native mussel *Perna viridis* and non-native *M. edulis* were infected. The infection of *M. edulis* at these sites suggests that other populations of this mussel are potentially threatened by 'new' parasite lineages in other regions of the world, at least where ecological conditions permit. *Marteilioides* reports confirmed by sequence data are restricted to South Korea and Japan, with non-sequenced records from Australia. *Paramyxa* (including *Paramyxoides*) has only been reported from Europe (Denmark, UK, France), and *Paramarteilia* only from the UK and (the original unsequenced type material) from northern France.

Patchy and low sampling effort explains at least some of these geographical observations. *Paramyxa* and *Paramarteilia* have only been studied at a small number of sites by very few researchers. An informed and more widely distributed sampling effort (including eDNA methods; Bass et al., 2015, and this study), is necessary and very likely to prove broader distributions for many paramyxid lineages. However, lineages that have been more intensively studied, for example *M. refringens* and *M. sydneyi*, are more likely to have been found without and within Europe, respectively, if they were present. Even so, there are many discoveries to be made, as demonstrated by the very recent finding of *M. refringens* in oysters *Crassostrea gigas* and *C. corteziensis* in Mexico (Grijalva-Chon et al., 2015) and *M. octospora* in Spain (Ruiz et al., 2016). Limited 18S data show *M. octospora* to be very closely related to *M. cochillia*, and while 18S data are not available for the Mexican *M. refringens*, their IGS rDNA sequences are only 0.3-2.2% dissimilar to *M. refringens* from a Spanish clam, *Chamelea gallina* (AM292652); with such similar sequences in the highly variable IGS region their 18S sequences are likely to be extremely similar or identical to the *M. refringens* sequence presented in Fig. Ch1-01.

There exist many potential paramyxid lineages for which inadequate microscopic or molecular data are available to establish their taxonomic affinity. For example, a *Marteilia* sp. has been observed at low prevalence (2% of 140 sampled) infecting the digestive gland of cultured rock oysters *Saccostrea forskali* in Chonburi Province, Thailand (Taveekijakarn et al., 2008). Similarly, unidentified *Marteilioides* sp. and *Marteilia* sp. have been observed (in the oocytes and digestive gland, respectively) of the Manila clam *Ruditapes philippinarum* (Itoh et al., 2005). These parasites are present at very low prevalence and have yet to be linked to any significant pathogenicity in their hosts. A further undescribed potential *Marteilia* sp. was also observed infecting the digestive diverticulum of the calico scallop, *Argopecten gibbus*, off Cape Canaveral, Florida, USA in 1988-89 (Moyer et al., 1993). This parasite resulted in the rapid decimation of the scallop population, but further attempts to collect material for ultrastructural analysis were unsuccessful. Although the taxonomic affiliations of this parasite remain unknown, a recent survey of calico scallop abundance in this area and the Gulf of Mexico shelf suggests the parasite is still present at both sites, with late stage infections common (Geiger et al., 2015). Further investigation is needed to ascertain the identity of the parasite and its effect upon the commercial viability of the scallop populations in these areas.

These reports prove that even if some paramyxids are much more geographically localised than is true for many protists (Bass & Boenigk, 2010), the diversity and distribution of the order as a whole is greater than is shown in Fig. Ch1-01 and Table Ch1-01. A more complete understanding of the distributions of these pathogens is increasingly important as new lineages

(often with economically significant effects) are being discovered, and known paramyxids are being found in new hosts and/or locations (e.g. *Marteilioides chungmuensis*, Itoh et al., 2004; *Paramarteilia canceri*, Feist et al., 2009; *Marteilia refringens*, Arzul et al., 2014; *M. sydneyi*, Adlard & Nolan, 2015). Human-mediated transport of these pathogens around the world could introduce them into areas in which they could become pathogenic if suitable environmental and/or lifecycle conditions arise. This also applies to other understudied parasites with similar, apparently restricted, geographical ranges, even though more intensive sampling facilitated by modern molecular screening methods shows these to be more widespread and diverse than previously though (e.g. haplosporids and mikrocytids; Hartikainen et al., 2014a,b).

Our new data and literature survey indicate that paramyxid lineages are being discovered on a regular basis, and perhaps with increasing frequency as knowledge of the group and methods to detect them improve. Initiatives such as the new 'Paramyxean Working Group' (http://paramyxeanworkingroup.org/) demonstrate that the international community has become more aware and interested in paramyxid research and there is a requirement and appetite for developing this neglected field. However, it remains difficult to estimate potential emergence and impact of paramyxids on animal hosts of human concern, and their even more hidden roles as parasites in diverse and interacting marine ecosystems because so little is known of their true diversity and distribution. In this study we provide some molecular tools for targeted detection of the full range of known paramyxids and environmental and organismal matrices. Our phylogenetic analyses provide an evolutionary context for understanding how the group evolved in terms of morphology, distribution and lifecycle. The recent

demonstrations that paramyxids occur in a wider range of hosts and environments than previously thought encourage us to study them and their pathogenesis in an ecological context, in addition to their individual effects on key host taxa. There is a need for greater genomic sampling of paramyxids, both to increase the power and phylogenetic (multi-gene) analyses of the group, and to better understand the nature of host-parasite interactions.

Table Ch1-T01. Review of paramyxids for which sequence data and/or microscopy evidence unambiguously identifies the lineage to at least genus. GenBank Accession numbers shown in bold are 18S sequences used in phylogenetic analyses (Fig. Ch1-01). Other sequences are either identical duplicates or a different gene region (Internal Transcribed Spacer 1 (ITS1), Intergenic Spacer (IGS) rDNA) from the same lineages. Bold geographical locations/citations represent findings confirmed by sequence data. Host names in brackets indicate PCR-positives where no histological evidence of infection is presented. Underlined entries indicate data generated in this study.

Parasite genus	Species	Host	Geographical Location	Citation	GenBank Accession
Paramyxa	*Paramyxa nephtys*	*Nephtys caeca*	Øresund, Denmark	Larsson & Køie (2005)	**KX259324** (partial 18S)
			Portland, UK		
		Environmental (water)	**Tamar estuary, UK**	**Ward et al. (2016)**	
	Paramyxa paradoxa	*Poecilochaetus serpens*	Banyuls-sur-Mer, France	Chatton (1911)	
	Paramyxa sp.	*Mytilus edulis*	**Tamar estuary, UK**	**Ward et al. (2016)**	**KX259326** (partial 18S)
	'*Paramyxa*' sp.	Environmental (water)	**Malaysian Borneo**	**Ward et al. (2016)**	**KX259325** (partial 18S)
	'*Paramyxa*' sp.	*Ostrea edulis*	**Tamar estuary, UK**	**Ward et al. (2016)**	**KX259323** (partial 18S)
	'*Paramyxa'* sp.	*Mytilus edulis* *Ostrea edulis*	**Tamar estuary, UK**	**Ward et al. (2016)**	**KX259327** (partial 18S)

Parasite genus	Species	Host	Geographical Location	Citation	GenBank Accession
Paramarteilia	*Paramarteilia canceri*	*Cancer pagurus*	Guernsey, UK Weymouth & Portland, UK South Kimmeridge Bay, UK	Feist et al., 2009	
	Paramarteilia orchestiae	*Orchestia gammarellus*	Taulé-Penzé, France	Ginsburger-Vogel et al. (1976)	
			Dale, UK **Weymouth, UK**	**Ward et al. (2016)**	
		Echinogammarus marinus	**Inverkeithing, UK** **Portsmouth, UK**	**Short et al. (2012a)**	**JQ673484** (partial 18S)
		Environmental (mollusc, crustacean incubations)	**Tamar estuary, UK**	**Ward et al. (2016)**	
	Paramarteilia sp.	Environmental (*Mytilus edulis* incubation)	**Tamar estuary, UK**	**Ward et al. (2016)**	**KX259320** (partial 18S)
Marteilioides	*Marteilioides branchialis*	*Saccostrea glomerata*	New South Wales, Australia	Anderson & Lester (1992)	
	Marteilioides chungmuensis	*Crassostrea ariakensis*	**Okayama, Japan** **Seomijn River, South Korea**	**Itoh et al. (2003)** **Yanin et al. (2013)**	AB110795 (18S) **GU132548(18S)**

Parasite genus	Species	Host	Geographical Location	Citation	GenBank Accession
Marteilioides	*Marteilioides chungmuensis*	*Crassostrea gigas*	**Tongyoung, South Korea**	**Yanin et al. (2013)**	GU132457(18S)
		Crassostrea nippona	**Japan**	**Iton et al. (2004)**	
	Marteilioides sp.	*Ruditapes philippinarum*	Hadong/Namhae, South Korea	Lee et al. (2001)	
			Tongyoung, South Korea	**Yanin et al. (2013)**	**GU132529(18S)**
Marteilia	*Marteilia christenseni*	*Scrobicularia piperata*	Marennes-Oléron, France	Comps (1983)	
	M. cochillia	*Cerastoderma edule*	**Ebro Delta, Catalonia, Spain**	**Carrasco et al. (2013)**	KF314809 (IGS)
			Ría de Arousa, Galicia, Spain	**Villalba et al. (2014)**	**KF278722(18S)**
	Marteilia cochillia	*Cerastoderma edule*	**Ebro Delta, Catalonia, Spain**	**Carrasco et al. (2013)**	KF314809 (IGS)
			Ría de Arousa, Galicia, Spain	**Villalba et al. (2014)**	**KF278722 (18S)**
	M. lengehi	*Saccostrea cucullata*	Bandar-Lengeh, Iran Queshm, Iran	Comps (1976)	

Parasite genus	Species	Host	Geographical Location	Citation	GenBank Accession
Marteilia	*M. octospora*	*Solen marginatus*	**Ría de Arousa, Galicia, Spain**	**Ruiz et al. (2016)**	KU641125(IGS, partial 18S) KU1641126 (ITS1)
	M. refringens	*Ostrea edulis*	Bassin d'Archachon, France	Grizel et al. (1974)	
			Île d'Oléron, France	**Berthe et al. (2000)**	**AJ250699 (18S)**
			Corsica, France	Pichot (2002)	
		Mytilus edulis	Brittany, France	Comps et al. (1975)	
			Brittany, France	**Berthe et al. (2000)**	**AJ250699 (18S)**
			Tamar estuary, UK	**Bignell et al. (2011)**	
		Mytilus galloprovincialis	Venice, Italy	Comps et al. (1982)	
			Galicia, Spain	Villalba et al. (1993)	

Parasite genus	Species	Host	Geographical Location	Citation	GenBank Accession
Marteilia		*Mytilus galloprovincialis*	**Istrian Peninsula, Croatia**	Zrnčić et al. (2001)	
			Ebro Delta, Catalonia, Spain	**Carrasco et al. (2008)**	
			Campania, Italy	**Carella et al. (2010)**	AB534169-70 (ITS1)
			Corsica, France	**Arzul et al. (2014)**	
			Slovene Adriatic Sea, Slovenia	**Gombac et al. (2014)**	JQ898012-14 (ITS1)
		Ostrea stentina	**Monastir Bay, Tunisia**	**Elgharsalli et al. (2013)**	JX119018-22 (IGS)
	M. refringens				
		Chamelea gallina	**Bay of Palma, Mallorca, Spain**	**Lópes-Flores et al (2008a)**	AM282652 (IGS)
		Solen marginatus	**Huelva, Spain**	**Lópes-Flores et al (2008b)**	AM748037-41 (IGS)
		Ruditapes decussatus	**Thau Lagoon, France**	**Boyer et al. (2013)**	
		Xenostrobus securis	Galicia, Spain	Pascual et al. (2010)	
		Paracartia grani	**Marennes-Oléron, France**	**Audemard et al. (2001)**	

Parasite genus	Species	Host	Geographical Location	Citation	GenBank Accession
Marteilia	*M. refringens*	*Paracartia latisetosa*	**Diana Lagoon, Corsica, France**	**Arzul et al. (2014)**	
		(Crassostrea gigas)	**Sonora, Gulf of California, Mexico**	**Grijalva-Chon et al. (2015)**	JQ066723-4 (IGS)
		(C. corteziensis)			JQ066725-6 (IGS)
		(Acartia clausi)	Ebro Delta, Catalonia, Spain	Carrasco et al. (2007b)	AM504139 (IGS)
		(A. discaudata)			AM504140 (IGS)
		(A. italica)			AM504141 (IGS)
		(Euterpina acutifrons)			AM504137 (IGS)
		(Oithona sp.)			AM504138; AM504145 (IGS)
		Environmental (water, sediment)	**Tamar estuary, UK**	**Ward et al. (2016)**	
	M. sydneyi	*Saccostrea glomerata*	Queensland, Australia	Perkins & Wolf (1976)	
			Queensland, Australia	**Kleeman & Adlard (2000)**	AF159248 (ITS1)
			Pimpama River, Qld, Australia	**Itoh et al. (2014)**	**AB823742(18S)**

Parasite genus		Host	Geographical Location	Citation	GenBank Accession
Marteilia	*M. sydneyi*	*Nephtys australiensis*	**Hawkesbury River, NSW,Australia**	**Adlard & Nolan (2015)**	
	Marteilia sp. MC	*Ruditapes philippinarum*	**South Korea**	**Kang et al. (unpublished)**	**AB823743 (18S)**
	Marteilia sp.	*Mytilus edulis*	**China**	**Wang et al. (2012)**	**KX259319 (18S)**
	Marteilia sp.	Environmental (*Penaeus* tank water)	**Malaysian Borneo**	**Ward et al. (2016)**	**KX259318** (partial 18S)
Eomarteilia	*E. granula*	*Ruditapes philippinarum*	**Kangawa, Japan**	**Itoh et al. (2014)**	**AB826587 (18S)**
	PARAM-1	Environmental (water)	**Gulf coast, Florida, USA**	**Ward et al. (2016)**	**KX259322** (partial 18S)
	PARAM-2	Environmental (water)	**Cariaco Basin, Venezuela**	**Edgcomb et al. (2011)**	**GU824205 (18S)**
		Environmental (water)	**Gulf coast, Florida, USA**	**Ward et al. (2016)**	**KX259321** (partial 18S)

Chapter Two:

‘Infection-Detection’: A study of *Marteilia pararefringens* infecting wild blue mussels *Mytilus edulis* in the Tamar Estuary, UK

Georgia M. Ward, Stuart Ross, John P. Bignell, Stephen W. Feist & David Bass

Chapter Description:

Marteilia spp. parasites are of great concern to bivalve aquaculture, particularly in Europe. Until recently *Marteilia* sp. infecting flat oysters *Ostrea edulis* and *Mytilus* spp. mussels was known as a single entity, *M. refringens*, comprising two distinct first internal transcribed spacer (ITS1) genotypes, 'O-Type' and 'M-Type', with mortalities only linked with 'O-Type' in oysters. Multigene analyses by Kerr et al. (2018) (see Appendix II) showed these two genotypes to be distinct species, and described *M. pararefringens,* 'M-Type', the only *Marteilia* species present in bivalves in northern Europe, where it exclusively infects mussels. Both genotypes have been treated as a single entity for many years, and so there is much confusion over the differences between *M. refringens* and *M. pararefringens* infection, and differences in the pathology and prevalence of both parasites in both bivalve hosts.

This study builds upon previous studies of *Marteilia* infecting mussels in the Tamar estuary, and the work presented in Kerr et al. (2018; Appendix II). The sampling site and individual *M. edulis* samples used in this study are those used in Chapter One, and the results of high-throughput sequencing of the diagnostic ITS1 region (presented in Kerr et al., 2018) show that only *M. pararefringens* is the only *Marteilia* species present in this population. Studies to date have focused on populations in geographical areas where both *M. pararefringens* and *M. refringens* are known to occur, or have utilised claire pond systems where parasite infection dynamics are known to differ greatly from estuarine systems. This chapter employs a range of PCR-based molecular assays for the detection of *M. pararefringens* in DNA and cDNA from *M. edulis*, paired with

complementary microscopic analyses to determine the efficacy of histopathology as a tool for the detection of early stages of *M. pararefringens* infection in mussels. The rate of infection within the Tamar population is low, with a single infection identified by *in situ* hybridisation that was not observed during routine histopathology. In this individual, early parasite stages were identified using ISH in the gill epithelium, consistent with previous findings of *M. 'refringens'* in *Mytilus galloprovincialis* in Alfacs Bay, Catalonia, Spain (Carrasco et al., 2008). However previous studies have failed to link these early infective stages to *Marteilia* genotypes (now distinct species), and so this study represents the first confirmation of *M. pararefringens* early life stafes outside of normal sites of infection, and highlights the inadequacy of histopathology-based protocols for the detection of nascent infections.

Author's Contribution:

GMW performed all DNA extractions, PCR screens and *in situ* hybridisation reactions, including contribution to the preparation of amplicons for Illumina sequencing as presented in Kerr et al. (2018).

Introduction

Marteilia (Rhizaria, Ascetosporea, Paramyxida) is a genus of protozoan parasites of marine molluscs. Most species are known as parasites of bivalves in Europe: *M. refringens* and *M. pararefringens* are both able to infect *Mytilus* spp. mussels and the flat oyster *Ostrea edulis*; *M. cochillia* infects and causes mortalities in the cockle *Cerastoderma edule* (Carrasco et al., 2013), and *M. octospora* infects the razor clam *Solen marginatus* (Ruiz et al., 2016). Two further species, *M. christenseni* (Comps, 1983) and *M. lengehi* (Comps, 1976) were described from hosts *Scrobicularia piperata* and *Saccostrea cucculata* respectively, though no confirmed observation of either parasite has been made since and so little is known about their effect on the host, and no molecular data exist for either species.

Both *M. refringens* and *M. pararefringens* are able to infect oysters and mussels, however only *M. refringens* has been proven to cause mass mortalities in oysters (Kerr et al., 2018). Oyster mortalities associated with *M. refringens* are so economically significant that the parasite is listed as a notifiable disease under World Organisation for Animal Health (OIE) legislation (OIE, 2018).

Outside of Europe, *M. sydneyi* is the causative agent of QX disease, responsible for severe mortalities in the Sydney rock oyster *Saccostrea glomerata* (Perkins & Wolf, 1976). A recent environmental DNA study and 18S phylogeny of the order Paramyxida revealed the existence of a number of uncharacterised *Marteilia* sequence types both in bivalve tissues and

environmental water samples (Ward et al., 2016; Chapter One of this thesis). The study also renamed *M. granula*, recently described from the Manila clam *Ruditapes philippinarum* in Japan (Itoh et al., 2014), *Eomarteilia granula* after phylogenetic analyses including all known paramyxid 18S sequence types placed this species as distinct from all other known *Marteilia* sequence diversity, and basal to all known paramyxids.

The type species of the genus *Marteilia*, *M. refringens*, was first described infecting and causing mortalities in flat oysters *O. edulis* in Aber Wrach River, Brittany, France, in the 1970s (Grizel et al., 1974). This was followed by the description of a closely related parasite, *M. maurini*, infecting Mediterranean mussels *Mytilus galloprovincialis* transported to France from Venice Lagoon, Italy (Comps et al., 1982). *M. refringens* and *M. maurini* were morphologically almost indistinguishable, with the only discernible difference considered to be the size and shape of haplosporosomes, and apparent host preference (Comps et al., 1982; Villalba et al., 1993). Prior to the description of *M. maurini,* parasites recognised as *M. refringens* were reported infecting *Mytilus* spp. and *O. edulis* in estuaries in Brittany, France (Tigé & Rabouin, 1976; Comps & Joly, 1980), and *M. galloprovincialis* in Spain (Villalba et al., 1993).

The availability of the first molecular data for *M. refringens* and *M. maurini* showed the two species to have identical small subunit rRNA genes (Le Roux et al., 2001). Further examination of a large number of infected individual *Mytilus* spp. and *O. edulis* concluded that the host specificity and subtle ultrastructural differences between *M. refringens* and *M. maurini* were invalid as criteria for distinction between the two parasites, and so the two species were

synonymised under the name *M. refringens*. (Longshaw et al., 2001; Balseiro et al., 2007).

Our understanding of *M. refringens* diversity continued to be complicated by the recognition of two distinct 'types', distinguished by the sequences of the faster evolving internal transcribed spacer (ITS1) ribosomal gene region. Dubbed 'O-Type' and 'M-Type', as a result of the supposed specificity of each type to oyster or mussel hosts respectively, this nomenclature is also misleading. 'O-Type' has been shown to also infect mussels, and vice-versa (Novoa et al., 2005); co-infections of both types in the same host have also been reported (Le Roux et al., 2001; López-Flores et al., 2004).

A recent molecular phylogenetic study utilising a multigene dataset and high-throughput sequencing of the ITS1 and intergenic spacer (IGS) regions from infected oysters and mussels from across Europe concluded the two 'types' of *M. refringens* represent distinct species with different geographic distributions (Kerr et al., 2018). 'O-type', now *M. refringens*, was absent from all screened oysters and mussels collected in northern Europe (SW England, west Norway, north-west Sweden) as part of this study, and from subsequent occurrences of *Marteilia* in Belfast Lough and Dundrum Bay in Northern Ireland. Only 'M-type', now *M. pararefringens,* was recovered in these areas, and only from mussels *M. edulis* and not oysters *O.* edulis. No mortalities were reported in any population, either as presented in Kerr et al. (2018) or as recorded by governmental Competent Authorities (Centre for the Environment, Fisheries and Aquaculture Science, Fish Health Inspectorate Quarterly Report, June 2017).

The description of *M. pararefringens* as a taxonomically distinct entity to *M. refringens* has important political implications. *M. refringens* is a pathogen listed as notifiable to the OIE and is also subject to European Union legislation (EU Council directive EC 2006/88) as a result of its economic effects on affected oyster populations. Any occurrence of *M. refringens* infecting any host must be immediately reported to the local Competent Authority, and trade restrictions are imposed. As such, to date all occurrences of *Marteilia* spp. in mussels in northern Europe have been subject to regulation, despite no mortality events attributed to *Marteilia* being reported in this geographic area. *M. pararefringens*, which can be distinguished from *M. refringens* using the diagnostic molecular signatures as presented by Kerr et al. (2018), is not subject to such legislative action.

Despite the economic importance of *Marteilia* spp. parasites in Europe and elsewhere, much remains unknown about their life cycles. Copepods and other zooplankton species have been implicated as alternative hosts by PCR (e.g. Carrasco et al., 2007b), though infection with *M. pararefringens* has been confirmed only in the Calanoid copepod *Paracartia* by *in situ* hybridisation (Arzul et al., 2014) in claire pool systems in France. Experimental transmission of *M. pararefringens* from infected copepods to oysters has been unsuccessful (Audemard et al., 2002), and Arzul et al. (2014) showed that incidence of the parasite in copepods as confirmed by PCR coincided with a steep decline in PCR presence in *Mytilus* at the same site. *Marteilia* was not present in any *O. edulis* sample collected at the same site.

Studies on the infection of *Mytilus* spp. mussels with *Marteilia* have centred largely on areas of Europe with extensive mussel culture, particularly Galicia, NW Spain (Villalba et al., 1993; Robledo & Figueras 1995), Alfacs Bay, Catalonia, Spain (Carrasco et al., 2007b;) and Corsica, France (Arzul et al., 2014). However, confusion over the delineation of the *Marteilia* species infecting mussels in southern Europe, and inconsistent or inadequate approaches to identification (such as the use of microscopic techniques without complementary sequencing of informative gene regions) means little is understood about the differences between *M. refringens* and *M. pararefringens* infections in mussel hosts.

This study focuses on the detection of early stages of *M. pararefringens* infecting blue mussels *Mytilus edulis* collected from Cremyll Ferry on the River Tamar estuary, SW England, UK. Mussel populations at this site and others in the Tamar estuary have been monitored for a range of pathogens as part of long-term studies by the Pathogens and Molecular Systematics group at the Centre for the Environment, Fisheries and Aquaculture Sciences (Cefas), with *Marteilia* known to have been present within these populations since at least 2006 (Bignell et al., 2011) with no associated mass mortality events. Prevalence of the parasite is typically low using routine histological observation, however PCR screens of mussel tissues using paramyxid- or *Marteilia*-specific primer sets indicates a larger number of potentially infected individuals (Ward et al., 2016; Kerr et al., 2018). Nascent infections are more easily missed during routine microscopic examination, particularly when a host response is not elicited (Carrasco et al., 2008). However as asserted by Burreson (2008), infection cannot be inferred from PCR alone, and while amplification of parasite

sequence types from DNA extracted from bivalve tissue may result from an established infection, it may also be the result of non-viable cells or non-infective parasite stages. Use of a cDNA PCR template, generated from RNA extracted from bivalve tissue, gives an indication of the transcriptional activity (and therefore viability) of cells present within the tissue (Logares et al., 2014). Here we compare a range of molecular techniques, including PCR using both DNA and cDNA templates, with traditional histopathology and a robust, well-tested *in situ* hybridisation protocol to determine the true prevalence of *Marteilia pararefringens* in the Tamar estuary, and assess the merits of molecular-led disease screening protocols for aquatic animal health.

Materials & Methods

Sample Collection and Preservation

Mussels *Mytilus edulis* were collected from naturally occurring wild populations at sites along the River Tamar estuary monthly between November 2012 and August 2013 (see Table Ch2-T02 for sampling dates and sites, and Fig. Ch2-01 for the locations of these sites). For use in this study, 153 *Mytilus* from Cremyll Ferry were collected in July 2013 and processed as in Ward et al. (2016), ensuring material was available from all individuals for light microscopy (histology and *in situ* hybridisation; cross-sections including digestive gland, mantle and gill tissues), transmission electron microscopy (TEM), and DNA and RNA extraction (flash-frozen mantle and digestive gland tissue).

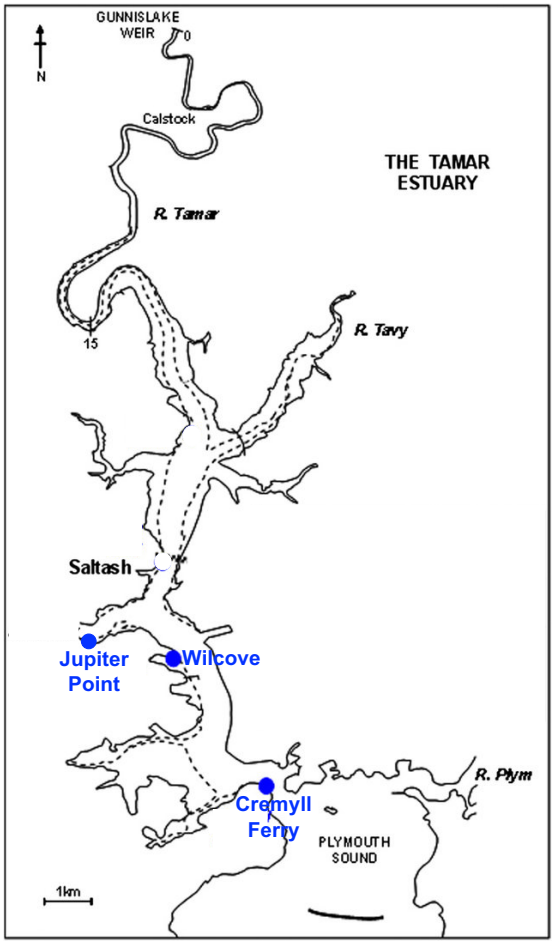

Figure Ch2-01. Map showing the location of sampling sites within the Tamar estuary, southwest England, United Kingdom. Adapted from Bignell et al. (2011)

Nucleic Acid Extraction and Processing

Flash-frozen tissues were defrosted into RNALater (Qiagen, Germany). DNA was extracted from mantle and digestive gland tissues separately from each individual using the 96-well DNEasy Blood and Tissue Kit (Qiagen).

RNA was extracted from both mantle and digestive gland tissues of individuals which were PCR-positive for *Marteilia pararefringens* based on a DNA template using the EZNA Mollusc RNA Kit (Omega Biotek, USA). Isolated RNA was screened for contaminating DNA using eukaryote-wide V9 PCR and quantified using the Qubit RNA-BR assay kit (Invitrogen, USA). 50 ng was then used to synthesise single-stranded cDNA using the iScript cDNA Synthesis Kit

(Promega, USA). RNA was also extracted from an additional, PCR- and histology-negative specimen (sample ID 100) for use as a control.

PCR and Sequencing

DNA extracted from the mantle and digestive gland tissues of all 156 *M. edulis* was screened using two nested primer sets: paramyxid-specific 18S primer set B (Ward et al., 2016; Chapter One) ('Paramyxid B Nest' in Table Ch2-03) and the ITS1-targeted MartDBITS primer set of Kerr et al. (2018), targeted at lineages showing high 18S sequence identity to European *Marteilia* spp. ('MartDBITS nest' in Table Ch2-03).

Three single-round PCR strategies were also employed to screen all mantle and digestive gland tissues: the second-round primers only of Paramyxid B and MartDBITS ('Paramyxid B single' and 'MartDBITS single' respectively in Table Ch2-02) and the *Marteilia* genus-specific 18S SS2/SAS2 primer set of Le Roux et al. (1999) ('SS2/SAS2 single' in Table Ch2-03).

cDNA prepared from PCR-positive (DNA template) individuals was screened using all three single-round PCR strategies.

PCR products were visualised on 2% agarose-TAE gels stained with GelRed (Biotium, USA), and resultant amplicons unidirectionally Sanger sequenced using the appropriate forward primer. Additionally, ITS1 amplicons from *Marteilia* histology-negative (TAM-3) and histology-positive (TAM-4) individuals were pooled separately, sequencing libraries prepared using the TruSeq PCR-Free Sample Preparation Kit (Illumina) and libraries sequenced on an Illumina

MiSeq as presented in Kerr et al (2018). This was to demonstrate that the only *Marteilia* sp. present in these mussels was *M. pararefringens*.

Digestive gland tissues from all mussels from July 2013 were genotyped using the Me15/Me16 primer set of Inoue et al. (1995) to determine if the individuals were *Mytilus edulis, M. galloprovincialis*, or hybrids of the two species, which is known to occur in southwest England. Amplicons produced using this PCR are short and similar in size (180bp and 126bp for *M. edulis* and *M. galloprovincialis* respectively, with both bands present in hybrid individuals), and so PCR products were visualised on 4.5% agarose-TAE gels stained with GelRed.

Histology and In Situ *Hybridisation (ISH)*

Slides including digestive gland, mantle and gill tissue were prepared for histology as in Ward et al (2016), and screening of samples for the presence of *Marteilia* spp. sporangia performed by pathologists at Cefas using a Nikon Eclipse E800 light microscope (Nikon, UK).

ISH was carried out on selected histology- and/or PCR-positive individuals using the *Marteilia* 18S-targeted 'Smart-2' probe developed by Le Roux et al (1999) (samples highlighted using red boxes in Table Ch2-T02). Digoxigenin (DIG)-labelled probes were synthesised by PCR in 100 µl volumes with a final concentration of 1x Promega colourless buffer, 2.5 mM $MgCl_2$, 20 µM PCR DIG labelling mix (Roche, Switzerland), 0.5 µM of each primer, 0.5 U of Promega GoTaq and 6 µl of template DNA from a histology-positive individual. Cycling conditions were as detailed in Le Roux et al (1999) for PCR using the SS2/SAS2 primer set. From each individual, 10 serial sections were mounted

on poly-L lysine slides. One slide was used for a negative control reaction for each individual (hybridisation buffer contained no probe). Deparaffinised slides were rehydrated and treated with Proteinase K (45 µg/ml) for 20 minutes at 37 °C in a humid chamber. Proteolysis was terminated by incubation in distilled water, and slides subsequently rinsed in ice-cold 12% acetic acid for 45 seconds, dehydrated in IMS and rinsed in 2X saline sodium citrate (SSC) solution for 1 min with agitation. Sections were overlaid with hybridisation buffer (4X SSC, 50% formamide, 1X Denhardt's solution, 10% dextran sulphate, 250 µg/ml single-stranded salmon sperm DNA) containing 50ng probe. Slides were heated to 94 °C for 10 mins and hybridised overnight at 42 °C. Sections were subsequently washed at room temperature in washing buffer (6M urea, 0.5X SSC and 20 µg/ml bovine serum albumin (BSA)) for 10 mins, followed by two further washes at 42 °C. Slides were blocked with 6% non-fat milk in Tris buffer (pH 7.5) and incubated with anti-DIG antibodies conjugated with alkaline phosphatase (1:300 in Tris pH 7.5) for 1 hour in darkness in a humid chamber. Reactions were then developed with 20 µl/ml nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (NBT/BCIP) (Roche, Germany) in Tris pH 9.5 in darkness until staining developed, or for 1 hour where staining was not readily visible. Sections were rinsed in Tris pH 9.5 and counterstained with 0.5% Bismarck Brown Y in 30% ethanol and dehydrated in IMS before they were coverslipped with Eukitt (Sigma Aldrich, Germany). Sections were examined under light microscopy for the presence of *Marteilia* life stages in any tissue type.

Results

As shown in Table Ch2-01, of 156 *M. edulis* collected at Cremyll Ferry in July 2013, *Marteilia* life stages were observed by routine histopathology in 4 individuals (2.56%). The indicated prevalence of *M. pararefringens* using nested and non-nested PCR strategies using DNA templates was 16.0-17.9% and 9.6-10.9%, respectively. Single-round screens of cDNA templates (from individuals PCR-positive using DNA templates) indicated a prevalence of 3.85%. *In situ* hybridisation, applied to selected samples with varying levels of molecular 'signal', indicated the prevalence of *M. pararefringens* to be 3.2%.

Table Ch2-01. Summary of the prevalence of *Marteilia pararefringens* in *Mytilus edulis* collected at Cremyll Ferry, Tamar estuary, UK in June 2013, as indicated by histopathology and molecular assays.

Detection Method	**Indicated Prevalence**
Histopathology (single slide)	2.56%
Nested PCR, DNA template (60 cycles)	16.0-17.9%
Single-round PCR, DNA template (30 cycles)	9.62-10.9%
Single-round PCR, cDNA template (30 cycles)	3.85%

Molecular Typing of Host and Parasite

Genotyping PCRs of 156 *Mytilus* collected in July 2013 and used for this study showed all sampled individuals to be *Mytilus edulis*. Similarly Illumina sequencing of pooled ITS1 amplicons from all histology-negative and histology-positive mussels confirmed that the only *Marteilia* species DNA present in these samples was *M. pararefringens*.

Histology Screening of Mytilus edulis *Tissue Sections*

For all mussels collected between November 2012 and August 2013, a single tissue section, comprising gill, mantle and digestive gland tissues and stained with haematoxylin and eosin was screened using light microscopy for the presence of *Marteilia* life-stages. Infection intensity was determined qualitatively using the criteria set out by Villalba et al. (1993): no infection (NI; no parasite observed), light infection (LI; parasite stages confined to stomach epithelium or primary ducts), moderate infection (MI; "a few" digestive tubules infected) or heavy infection (HI; infection spread through digestive diverticulata, infecting more than 10% of digestive tubules visible in a histological section).

Table Ch2-T02. Results of histopathological screens for *Marteilia* life-stages in *Mytilus* sp. mussels collected at sites within the Tamar estuary between November 2012 and August 2013. Infection level is qualitatively determined using the criteria stated by Villalba et al. (1993)

Year	Month	Site	Number sampled	***Marteilia* infections observed (Infection level)**	**Prevalence of *Marteilia* (%)**
2012	November	Jupiter Point	32	0	0
2013	January	Jupiter Point	150	0	0
	February	Wilcove	120	3 (3x HI)	2.50
	March	Wilcove	121	0	0
	May	Wilcove	60	0	0
	June	Cremyll Ferry	153	3 (2x HI; 1xMI)	1.96
	July	Cremyll Ferry	156	4 (3x HI; 1x LI)	2.56
	August	Wilcove	60	0	0

The results of this are shown in Table Ch2-T02. The parasite was only observed in samples collected in 3 months: February, June and July 2013, and was detected at the Cremyll Ferry and Wilcove sites, consistent with Bignell et al. (2011). Prevalence was highest at Cremyll Ferry in July 2013 (the sample set used for more intensive molecular and microscopic screening), reaching 2.56%, however prevalence of the parasite also reached 2.50% in February 2013 at Wilcove. Prevalence was lower in June 2013 (1.96%).

PCR Screening of Mytilus edulis *DNA*

Table Ch2-T03 summarises in detail the results of microscopy screens and PCR assays applied to the 156 mussels collected in July 2013. 28 were PCR-positive for *Marteilia pararefringens* using the nested, paramyxid-specific 18S primer set; 25 of these were also PCR-positive using the nested *Marteilia*-targeted ITS1 primer set. A single specimen produced amplicons from both digestive gland and mantle tissues using each primer set, and a further sample was PCR-positive from mantle tissue only with each primer set. The remainder of nested PCR-positives were from digestive gland tissue.

The second-round primer sets of the 18S- and ITS1-targeted PCR strategies were also used as single round PCR. A further single-round PCR strategy, SS2/SAS2, also targeting the 18S rRNA, was also used. The second-round 18S-targeted primers produced 15 amplicons (all from digestive gland tissue). The same 15 samples were also PCR-positive using the second-round ITS1 primers. The SS2/SAS2 primer set also amplified the same 15 samples, and two additional digestive gland specimens.

PCR Screening of Mytilus edulis *cDNA*

RNA was extracted from all samples which were PCR-positive from screens of DNA, and an additional histology- and PCR-negative sample. cDNA generated from this RNA was screened using only single-round strategies, all of which used 30 cycles of amplification: the second round primers of the 18S-targeted paramyxid primer set, the second round primers of the ITS1-targeted *Marteilia* primer set, and the SS2/SAS2 primer set. Of 28 previously PCR-positive samples (17 previously positive by non-nested PCR), 6 were PCR-positive using the general paramyxid 18S primer set, 7 using the *Marteilia*-specific ITS1 primer set and 5 using the SS2/SAS2 primer set.

In situ *hybridisation*

A total of 5 individuals were selected for *in situ* hybridisation using the SMart2 probe developed by Le Roux et al (1999). Of these, one showed a heavy *M. pararefringens* infection (HI; Sample 151) during histology screening, and one a light infection (LI; Sample 119). In both of these samples, staining of probe-labelled *Marteilia* tertiary cells in the digestive tubules and mantle connective tissue were observed in all ISH slides Fig. Ch2-02A, B), except negative controls. No infection was observed in the remaining three samples in initial histology screens. Sample 147 was PCR-positive using all assays from DNA and cDNA templates, and staining of small cells similar in size and shape to *Marteilia* life-stages reported by Carrasco et al. (2008) were observed in the gills of 6/9 ISH slides (Fig. Ch2-02C,D). Sample 133 was PCR positive in all assays using a DNA template, but negative in those using a cDNA template. No staining of cells was observed in any ISH slide. Sample 100, negative in all assays, also showed no staining of cells in any ISH sample.

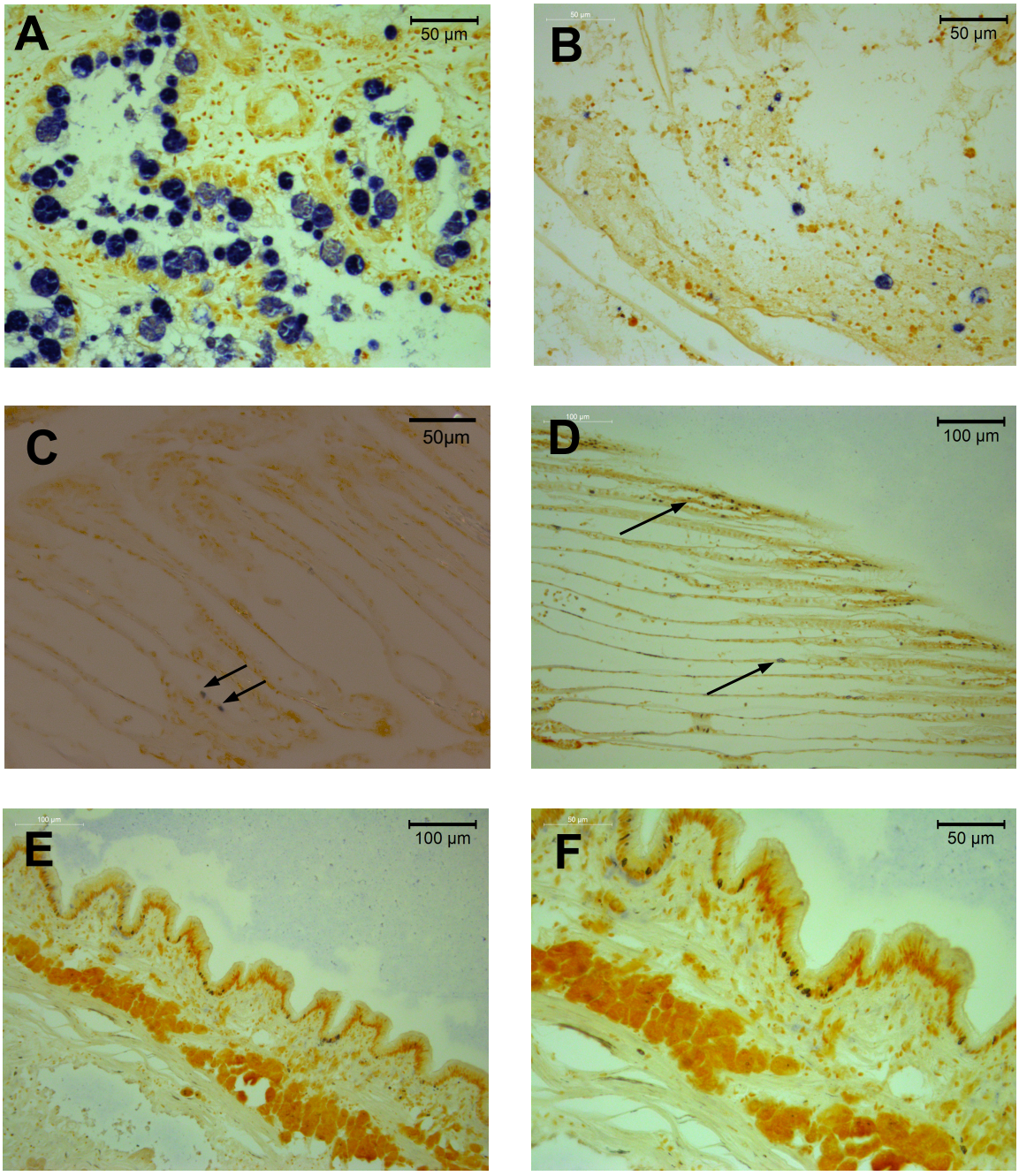

Figure Ch2-02. *In situ* hybridisation light micrographs showing DIG-stained *Marteilia* life stages (purple cells) in *Mytilus edulis* tissue. **A.** *Marteilia pararefringens* infecting the secondary digestive tubules of a specimen in which *Marteilia* life stages were readily observable in routine histopathology screens (infection level (HI). **B.** *M. pararefringens* in the mantle connective tissue of the same specimen as A. **C, D.** Early life-stages of *M. pararefringens* in the gill epithelium of a *M. edulis* individual in which no parasite cells were observed in routine histopathology (arrows). **E, F.** DIG-stained *M. pararefringens* life-stages in the mantle epithelium of the same specimen as images C and D. All PCR assays using DNA and cDNA templates from digestive gland tissue of this mussel were positive.

Table Ch2-T03. Summary of the results of microscopy (histology and *in situ* hybridisation (ISH)) and PCR assays for the detection of *Marteilia pararefringens* in tissues of *Mytilus edulis* collected at Cremyll Ferry, Tamar estuary, SW United Kingdom in July 2013. Sample ID indicates the unique identification number allocated to each of the 156 individuals upon collection. Those individuals in which no microscopic or molecular signal of the presence of *M. pararefringens* was found have been omitted. Numbers in ISH column indicate the number of slides in which *M. pararefringens* life stages were observed out of a total of 9 screened. Cells shaded black represent PCR or microscopy detection of *M. pararefringens,* grey represent samples which not were screened using selected assays, and unshaded cells indicate no detection of *M. pararefringens*. Samples highlighted in red were analysed using all light microscopy and PCR assays.

			DNA PCR										cDNA PCR					
	Histology		Paramyxid B nest		MartDBITS nest		Paramyxid B single		MartDBITS single		SS2/SAS2 single		Paramyxid B single		MartDBITS single		SS2/SAS2 single	
Sample ID		ISH	DG	M	DG	M	DG	M	DG	M	DG	M	DG	M	DG	M	DG	M
10	■	▒	■		■		■		■		■		■	▒	■	▒	■	▒
16		▒		■		■							▒		▒		▒	
17		▒	■		■									▒		▒		▒
23		▒	■		■									▒		▒		▒
29		▒	■		■						■			▒		▒		▒
38		▒	■		■						■			▒		▒		▒
44		▒	■		■									▒		▒		▒
48		▒	■		■									▒		▒		▒
67		▒	■		■		■		■		■			▒		▒		▒
73		▒	■		■		■		■		■			▒		▒		▒
78		▒	■		■									▒		▒		▒

Sample ID	Histology	ISH	DNA PCR										cDNA PCR					
			'Paramyxid B' nested		'MartDBITS' nested		'Paramyxid B' single		'MartDBITS' single		SS2/SAS2 (single)		'Paramyxid B' single		'MartDBITS' single		SS2/SAS2 (single)	
			DG	M	DG	M	DG	M	DG	M	DG	M	DG	M	DG	M	DG	M
82			■		■		■				■							
84			■		■		■		■		■							
89			■															
95			■		■		■		■		■							
100		0/9																
105			■															
118			■		■		■		■		■							
119	■	9/9	■		■		■		■		■		■		■		■	
120			■															
131			■		■		■		■		■							
133		0/9	■		■		■		■		■							
138	■		■		■		■		■		■		■		■		■	
143			■		■		■		■		■		■		■			
147		6/9	■		■		■		■		■		■		■		■	
150			■		■		■		■		■				■			
151	■	9/9	■		■		■		■		■		■		■		■	

Discussion

Marteilia pararefringens has been present in *Mytilus* populations within the Tamar estuary since at least 2006 (Bignell et al., 2011). The populations studied are all naturally occurring, and not located close to any site where *Mytilus* spp. mussels are commercially exploited, and so no individuals are known to have been translocated into the study site. During the duration of this study, the prevalence of the parasite has remained low, with no mass mortality events associated with its presence, though individual mortalities attributed to the parasite were observed. Our findings are consistent with this. Prevalence peaks in early summer (June 2013, 1.96%; July 2013 2.56% at Cremyll Ferry), in line with *Marteilia* spp. infections in mussels in Spain (Villalba et al., 1993; Carrasco et al., 2008).

Prevalence of parasite DNA as indicated by PCR was highest using more sensitive, nested strategies (16.03-17.95%), and still significantly higher than histology screens when using single-round strategies (9.62-10.90%). Parasite prevalence in cDNA is much lower (3.2-4.5%) than DNA, suggesting the majority of amplifications from DNA templates do not represent the presence of viable, transcriptionally active cells, and therefore are unlikely to indicate an established or nascent infection. Ward et al. (2016) showed *Marteilia* 18S sequence types to be present in sterile seawater in which infected mussels have been incubated overnight, as well as in the water column and sediment surrounding these mussels. *M. refringens* spores are also known to be expelled in mussel faeces, however both experimental inoculation of mussels with *Marteilia* sporangia and cohabitation in tanks with infected individuals does not

result in *M. refringens* transmission (Berthe et al., 1998). It is likely this is also the case for *M. pararefringens*, and it is possible that the amplification of *Marteilia* sequence types from the tissues of mussels cohabiting in close proximity to other infected individuals results from passive ingestion of non-viable *Marteilia* cells or extracellular DNA via filter feeding. However Kerr et al. (2018) note that the prevalence of *M. pararefringens* by PCR in cohabiting oysters at sites in the Tamar estuary is very low (1/300 samples amplified using nested PCR, with no evidence of infection), so this passive transmission of *Marteilia* DNA is likely to only occur over very short distances.

The molecular prevalence of *M. pararefringens* in assays using an RNA-derived (cDNA) template is much lower, and close to the prevalence observed during routine histopathology. Just two additional individuals indicated the prevalence of viable *M. pararefringens* cells in all molecular assays, and following the application of *Marteilia*-specific ISH probes to one of these samples, stained cells similar in appearance to those observed by Carrasco et al. (2008) were observed in the gill epithelium in 6/9 slides. No host response was observed, and so this is likely to represent a newly-established infection. It should be noted that infection should not be inferred from the presence of parasite sequence types in RNA samples alone. While parasite cells may be present and transcriptionally active, this gives no information of the life-stage of such cells and whether they are capable of infecting host tissues. The status of parasite cells as either established infections or merely present in the host tissue sampled (for example by passive filter feeding) can only be determined by microscopic methods such as histology or ISH.

In situ hybridisation has proven a valuable tool in the elucidation of early infection stages of other paramyxid parasites. Kleeman et al. (2002) used the technique to identify stages of *M. sydneyi* infecting the gill and palp epithelia, and connective tissues of the digestive gland, palps and mantle of *Saccostrea glomerata*. Similar observations have been made using ISH of early developmental stages of *Marteilioides chungmuensis* in the epithelia of the gills, mantle and labial palps of *Crassostrea gigas* (Itoh et al., 2004). Carrasco et al. (2008) observed early stages of what was presumed to be *Marteilia refringens* in the gill epithelia of *Mytilus galloprovincialis,* though no molecular characterisation of the parasite was performed.

Studies focusing on the suitability of *in situ* hybridisation as a diagnostic method for the detection of paramyxid parasites have also shown promise. Choi et al. (2012) compare the efficacy of ISH and PCR for the detection of *Marteilioides chungmuensis* in *C. gigas* against histology, with their findings indicating that ISH, a microscopic approach using labelled molecular probes, has much greater sensitivity at detecting light infections than either microscopic or molecular method employed. In this case almost twice as many individuals were found to be infected using ISH compared to histology, however the majority of these were low-level infections with only early parasite stages present. Thébault et al. (2005) compared the efficacy of ISH and histology for the detection of *Marteilia refringens* in *Ostrea edulis* and found ISH to be the more sensitive technique, though the authors raise the point that perceived levels of specificity in one population and set of ecological conditions may not easily be extrapolated others.

As demonstrated in this study and the above publications, molecular-based methods including PCR and ISH are powerful tools for the detection of newly-established and low-level infections. There is a huge burden on reference labs to reliably diagnose disease in a large number of individuals in a relatively short space of time, however this requires highly trained, experienced individuals. The results of this study also suggest that the scale for determining intensity of infection of *M. refringens* in *Mytilus galloprovincialis* put forward by Villalba et al. (1993) is not sufficiently detailed to include all observable infection levels. The lowest infection "rating" on this scale ('light infection') is defined as the presence of parasite life-stages confined to the stomach epithelium or at most having reached the primary digestive ducts. The work contained herein, and the observation of *Marteilia* sp. in the gills of *Mytilus galloprovincialis* (Robledo & Figueras, 1995; Carrasco et al., 2008) and *Ostrea edulis* (Comps, 1970; Grizel et al. 1974), show that infection of tissues other than those mentioned on the scale of Villalba et al. (2003) is common, and though considered "unusual" (Carrasco et al., 2008) should not be discounted.

Though infection of the gill epithelium of both *O. edulis* and *Mytilus galloprovincialis* with *Marteilia* life-stages have been reported previously, in no case is it possible to determine whether the observed infection was M-type (*M. pararefringens*) or O-type (*M. refringens*). At present *M. refringens* and *M. pararefringens* cannot be distinguished from each other using any reliable morphological characteristics, and genomic data for both species remain unavailable. *M. refringens* is an OIE-listed pathogen, and so is subject to legislation limiting trade and movement of stocks from areas where the disease has been reported to disease-free locations. It is therefore important to make

appropriate distinctions between *M. refringens* and *M. pararefringens*, to which no restrictive legislature applies. As such it should now be a priority to increase our understanding of the genetic basis of supposed differences in pathogenicity between these two lineages, particularly as techniques such as whole genome sequencing and transcriptome analysis become more widely available and affordable. This may offer not only more robust methods for pathogen identification, but also offer greater insight into the interactions between parasite and host, and how these influence disease progression.

However the application of molecular methods for disease detection – such as the ITS1-specific PCR targeting diagnostic marker regions presented in Kerr et al. (2018) – followed up by microscopic methods including ISH and histopathology would allow the rapid assessment of the identity of *Marteilia* spp. present within populations. Through the application of complementary molecular typing and light microscopy techniques, applied to infections in all susceptible hosts, differences between the pathogenicity of *M. refringens* and *M. pararefringens* may become more apparent.

Chapter Three:

Detection and Characterisation of Haplosporidian Parasites of the Blue Mussel *Mytilus edulis,* including description of the novel parasite *Minchinia mytili* n. sp.

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Chapter Description:

This chapter focuses on the detection and diversity of haplosporidian parasites of the blue mussel, *Mytilus edulis*. Wild and farmed populations of *M. edulis* are common across Europe and indeed worldwide, with very few instances of mass mortality reported annually. Despite numerous previous reports of haplosporidian parasites infecting *Mytilus* spp. worldwide, only one species has ever been formally characterised, though was later shown to have been misidentified, and no sequence data are available from any of these reports. In this study, PCR screens of *M. edulis* tissue with general haplosporidian primers indicated a high prevalence of two distinct haplosporidian sequence types. Histopathology and *in situ* hybridisation techniques are used to provide morphological data for the first of these species, and to unequivocally link this morphology with sequence type. This species is formally described as *Minchinia mytili* on the basis of molecular data. Though no morphology associated with the second sequence type was observed in samples from the Tamar estuary, a specimen of *M. edulis* heavily infected with a novel haplosporidian parasite collected in Loch Spelve, Scotland, was found to match the same novel sequence type. In this chapter specific PCR assays for the detection of each sequence type have been developed, and both sequence types extended to cover nearly the complete 18S rDNA gene to provide as much sequence data as possible for robust molecular phylogenetic analysis. Diagnostic molecular markers are also identified for both species, and specific ISH probes have been developed for *M. mytili*.

Author's Contribution:

Georgia Ward contributed to the experimental design, including specific primer and *in situ* hybridisation (ISH) probe design. GMW also processed all DNA extractions, and performed all PCR screens and ISH reactions and phylogenetic analyses, and drafted and revised the manuscript for publication.

Abstract

The blue mussel *Mytilus edulis* is a major aquaculture commodity in Europe, with 168,000 metric tonnes produced in 2015. A number of abundant, well-characterised parasites of the species are known, though none are considered to cause significant mortality. Haplosporida (Rhizaria, Ascetosporea) is an order of protistan parasites of aquatic invertebrates, the best studied of which are oyster pathogens *Haplosporidium nelsoni* and *Bonamia ostreae*. While these species are well characterised within their hosts, the diversity, life-cycle and modes of transmission of haplosporidians are very poorly understood. Haplosporidian parasites have previously been reported from *Mytilus* spp., however the majority of these remain uncharacterised, and no molecular data exist for any species. In this study, we identify two novel haplosporidian parasites of *M. edulis* present in the UK. The first of these, observed by light microscopy and *in situ* hybridisation infecting the gills, mantle, gonadal tubules and digestive connective tissues of musels in the Tamar estuary, Devon, we describe as *Minchinia mytili* n. sp. on the basis of 18S sequence data. The second, observed infecting a single archive specimen collected in Mull, Scotland, infects the foot muscle, gills and connective tissues of the digestive gland. Sequence data places this parasite in an uncharacterised clade of sequences amplified from tropical bivalve guts and water samples, sister to *H. nelsoni*. Screening of water and sediment samples collected at the sample site in the Tamar estuary revealed the presence of both sequence types in the water column, suggesting host-free or planktonic life stages.

Introduction

The blue mussel *Mytilus edulis* Linneaus 1798 is of huge significance to aquaculture worldwide, with over 190,000 metric tonnes produced globally in 2015 (FAO, 2017). The majority of this was farmed in Europe (168,000 t), with France (61,000 t), the Netherlands (54,000 t) and the United Kingdom (20,000 t) the largest producers. Outside of Europe, the Atlantic coast of Canada is also a major producer (22,000 t in 2015). In addition to the value of this species to global aquaculture, as filter feeders mussels play an important role ecologically, filtering waterborne nutrients and particles, and their sessile lifestyle makes them ideal proxies for the detection of local environmental contaminants such as heavy metals and organic chemicals, as demonstrated by the United States' National Oceanographic and Atmospheric Administration (NOAA) Mussel Watch program (Kimbrough et al., 2008).

Continued interest in the economic exploitation of mussels for aquaculture has led to a number of attempts to establish baselines for population health, aiming in particular to shed light on the parasites and pathogens of the species, their significance and distribution (e.g. Figueras et al., 1991; Bignell et al., 2008). Such studies predominantly utilize histopathological methods, and have identified several highly prevalent, widely distributed parasites, including the copepod *Mytilicola intestinalis* and the gill ciliate *Ancistrum mytili*. Both of these are ubiquitous in *Mytilus* spp. populations across Europe, and though they can be linked to a loss of condition they are not thought to be associated with increased mortality in the host (Villalba et al., 1997). More recently, studies of

baseline population health have incorporated molecular screening of mussel tissues for specific parasite groups, allowing for the more sensitive detection of rarer, low-prevalence parasitic infections (e.g. Lynch et al., 2014).

Over recent decades, the protistan class Ascetosporea (Rhizaria, Endomyxa) has emerged as an important group of parasites of economically significant aquatic species. Ascetosporea is comprised of five orders, the most well known of which is the Haplosporida, which includes species known to infect molluscs, crustaceans and annelid worms in both marine and freshwater environments. The orders Mikrocytida, Paramyxida and Paradinida also include important invertebrate pathogens, though their diversity and host range are only very recently becoming apparent (Hartikainen et al., 2014b; Ward et al., 2016; Ward et al., 2018). The fifth order Claustrosporida remains very poorly characterized, comprising just two described species, *Claustrosporidium gammari* and *C. asellii*, with no molecular data available for either species (Larsson 1987; Cavalier-Smith & Chao, 2003b).

Haplosporida includes over 40 described species across four genera (*Haplosporidium, Minchinia, Bonamia* and *Urosporidium)*, and a number of undescribed species (Arzul & Carnegie, 2015). In addition, recent haplosporidian-targeted molecular studies using group-specific primer sets to probe environmental water and sediment (Hartikainen et al., 2014a) and bivalve gut samples (Pagenkopp-Lohan et al., 2016) have revealed significant uncharacterised molecular diversity within the order.

The most well-studied haplosporidian species are the oyster parasites *Haplosporidium nelsoni* and *Bonamia ostreae*, responsible for mass mortalities in *Crassostrea virginica* and *Ostrea edulis* respectively (Ford & Haskin, 1982; Robert et al., 1991). Economic losses associated with *B. ostreae* infection are so significant that the parasite is currently listed as notifiable to the World Organisation for Animal Health (OIE), http://oie.int/en/international-standard-setting/aquatic-code (2017).

Urosporidium spp. parasitise free-living annelid worms and Platyhelminthes, themselves parasites of marine molluscs and crustaceans. *Minchinia* spp. have been reported only from marine molluscs (Ford et al., 2009), and *Bonamia* spp. from oysters (Engelsma et al., 2014). The most speciose genus, *Haplosporidium*, is comprised of species parasitising molluscs, crustaceans and annelid worms in both marine and freshwater environments (Arzul & Carnegie, 2014).

The morphology of haplosporidian spores has frequently been used to assign species to genera, with species belonging to the 'microcell' genus *Bonamia* previously presumed not to form spores (Perkins, 2000). Haplosporida form ovoid, walled spores without polar filaments or tubules, with an orifice at one pole. *Urosporidium* spp. produce unornamented spores with an internal flap composed of spore wall material covering the orifice. In *Minchinia* and *Haplosporidium* spp., the orifice is covered by an external hinged lid. Both genera form ornamented spores, though differ in the origin of this ornamentation: in *Minchinia* spp., ornamental extensions are formed of epispore cytoplasm, while the extensions of *Haplosporidium* spp. spores are composed

of spore wall material (Burreson & Ford, 2004). All *Urosporidium* and *Minchinia* species for which the spore structure are known are concordant with these criteria, but the only species of *Bonamia* known to form spores, *B. perspora*, produces spores matching the criteria for *Haplosporidium* (Carnegie et al., 2006). Further, lineages with the 'Haplo' spore structure do not form a monophyletic clade (Burreson & Reece, 2006; supported by more recent analyses by Hartikainen et al., 2014a, and Pagenkopp-Lohan et al., 2016), and are therefore unsuitable for defining *Bonamia* and *Haplosporidium*; even more so when the necessary taxonomic revisions have been made to *Haplosporidium.* It is likely that more genera must be created in order to resolve the paraphyletic *Haplosporidium*. However, with spore structure and molecular data currently lacking for the type species, *Haplosporidium scolopli*, it remains unclear which clade should be considered the true *Haplosporidium* (Arzul & Carnegie, 2015).

The life-cycles of haplosporidian parasites are also largely unknown, though evidence exists to suggest direct transmission between hosts in at least some non spore-forming *Bonamia* species, including *B. ostreae* (Engelsma et al., 2014). Numerous unsuccessful attempts have been made at direct transmission of *Haplosporidium nelsoni* and so the involvement of intermediate hosts in the parasite life-cycle is suspected, though none have been identified (Powell et al., 1999). The presence of a large diversity of haplosporidian sequence types in the water column and sediment may suggest either free-living transmissible stages, or the involvement of planktonic metazoans in haplosporidian life-cycles (Hartikainen et al., 2014a).

Haplosporidians have been reported in *Mytilus* spp. mussels on numerous occasions, however only two have ever been formally described: *Haplosporidium tumefacientis*, causing distinctive tumour-like lesions in the kidney and digestive tissues of *M. californiensis* in California, USA (Taylor, 1966), and *Haplosporidium mytilovum*, infecting the ovocytes of *M. edulis*, which was later reassigned to the microsporidian genus *Steinhausia* following microscopic examination of fresh material (Sprague, 1972). More recently haplosporidians have been observed infecting *Mytilus* spp. in Maine, USA (Figueras et al., 1991), southern France (Comps & Tigé, 1997) and the Atlantic coast of Canada (Stephenson & McGladdery, 2002). However insufficient morphological data were available in each case to formally describe any of these parasites, and no molecular data exist for any haplosporidian parasite of *Mytilus*.

This study combines traditional histopathological light microscopy with targeted, group-specific environmental and host-focused molecular probing and *in situ* hybridisation (ISH) to characterise a novel haplosporidian parasite infecting *M. edulis* in Devon, United Kingdom. A further novel haplosporidian is informally described from a single archive specimen of *M. edulis* from the west coast of Scotland.

Materials & Methods

Sample Collection & Processing

156 *Mytilus edulis* were collected from naturally-occurring populations the River Tamar estuary mouth near Cremyll Ferry, Devon, UK, in June 2013 and

incubated overnight (Hartikainen et al., 2014b) in sterile artificial seawater (ASW; Culture Collection of Algae and Protozoa (CCAP)) in batches of ten clustered according to sampling proximity. Transverse sections to include the digestive gland, gonad, gills and mantle were then fixed in Davidson's Solution for histological processing and examination, 2.5% glutaraldehyde for electron microscopy (EM), and 100% molecular-grade ethanol for molecular analyses, as in Ward et al. (2016). Subsequent processing for microscopy and DNA extraction were as described in Ward et al. (2016). Water samples were collected from three sites in the Tamar estuary (Cremyll Ferry, Wilcove and Neal Point), processed and DNA extracted as in Ward et al. (2016). A further 153 *M. edulis* were collected from the same site in July 2013 and tissues fixed for microscopy as above. Digestive gland and mantle tissues were preserved in RNALater (Qiagen) for molecular analyses, as in Ward et al. (2016). As presented in Chapter Two, all mussels included in this study were genotyped using the Me15/Me16 primer set targeting the *Glu* gene of *Mytilus* spp. and frequently used to discriminate between *M. edulis, M. galloprovincialis*, *M. trossulus* and hybrids. In this case, all mussels collected during the study were *M. edulis*.

As part of an investigation into observed mortalities by Marine Scotland, 40 *M. edulis* were collected from a farmed population in Loch Spelve, Mull, Scotland in January 2011. Tissues were preserved for histology. DNA was later extracted from formalin-fixed material from a single specimen in which haplosporidian plasmodia were observed during histology screening, using the EZNA FFPE DNA extraction kit with the kit's standard xylene deparaffinisation protocol (Omega Biotek, Georgia, USA), and the identity of this specimen as *M. edulis*

confirmed using the *Glu*-targeted PCR described above. Identity of other individuals collected or present at the site could not be determined as samples were not available.

PCR and Sequencing

All PCR reactions were performed in 20 µl volumes consisting of 1X Promega Colourless Buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 µM each primer, 0.2 mg bovine serum albumin (BSA), 0.5 U GoTaq G2 (Promega, USA), and 1 µl template DNA. The sequences of all primers used in this study are shown in Table Ch3-T01. DNA extracted from *M. edulis* tissues from the Tamar estuary was screened using the nested, haplosporidian-specific primer set of Hartikainen et al. (2014a), targeting the V7-V9 variable regions of the 18S rRNA gene. Resultant amplicons were visualised on 2% agarose gels stained with GelRed (Biotium) and Sanger sequenced unidirectionally using primer V5fHapl. The two distinct haplosporidian sequence types ('Type 1' and 'Type 2') amplified from these tissues were aligned with all known haplosporidian sequence types using the MAFFT e-ins-I algorithm (Katoh & Standley, 2013). This alignment was used to design specific primer sets for each sequence type to be used for lineage-specific PCR screening of incubation and eDNA samples, and for the generation of ISH probes. The specificity of each primer set was tested by screening a panel of tissues infected with other haplosporidian species (*Bonamia ostreae, Haplosporidium nelsoni, H. edule, H. littoralis, H. costale* and *Minchinia mercenariae*), and uninfected, PCR-negative host DNA samples. In each case, an amplicon was produced only from templates known to be the intended lineage, as confirmed by Sanger sequencing. The first round general haplosporidian primer set of Hartikainen et al (2014a), C5fHap and

Sb1n, was used for the first round for both lineage-specific PCRs. PCR probing for Type 1 used primers Hap-M258f and Hap-M412r, and the following cycling conditions: 5 minutes denaturation at 95°C, followed by 30 cycles of 95°C for 1 minute, annealing at 63°C for 1 minute and extension at 72°C for 1 minute. Amplicons were extended by final incubation at 72°C for 10 minutes and stored at 4°C. Screens for Type 2 used primers Hap-E312f and Hap-E620r, and the same conditions as for Type 1, but with an annealing temperature of 64°C. The single haplosporidian-infected *Mytilus* collected from Loch Spelve, Scotland was screened using the Type 1 and Type 2 primer sets individually as a single-round PCR only (i.e. no nesting). Mussels collected in the Tamar estuary were screened once more by PCR separately using only Type 1 and Type 2 lineage-specific primers (i.e. not nested) to give an approximation of prevalence of molecular signal.

Prior to phylogenetic analysis, both haplosporidian SSU sequence types were extended by PCR to include the V2-V7 variable regions. To extend Type 1, first round PCR used primers HapGenFor33 and Hap-M412r, followed by a second round using primers HapGenFor84 and Hap-M412r. For Type 2, first round PCR used primers HapGenFor33 and Hap-E620r, followed by a second round using HapGenFor84 and Hap-E449r. All four PCRs used the following cycling conditions: 5 minutes denaturation at 95°C, followed by 40 cycles of 95°C for 30 seconds, annealing at 55°C for 1 minute and extension at 72°C for 1m 30s. Amplicons were extended by final incubation at 72°C for 12 minutes and stored at 4°C. Amplicons were then visualised on a 1.5% agarose-TAE gel stained with GelRed, and bidirectionally sequenced using second round primers. Low

quality base calls were removed before consensus sequences were formed from these reads and initial V7-V9 amplicons.

Phylogenetic analysis

All available full length haplosporidian 18S sequence types, and those covering the V5-V9 variable regions were downloaded from NCBI GenBank in August 2017. Sequences not overlapping this region, or without significant (>200bp) sequence overlap were excluded from analyses. Blastn searches of haplosporidian sequences against the GenBank database were used to identify uncharacterised (including environmental) sequences related to known taxa. The extended haplosporidian sequence types generated in this study were aligned with these using MAFFT version 7, e-ins-I algorithm (Katoh & Standley, 2013). The resulting alignment, using the closest known haplosporidian relatives ENDO-3 (Bass et al., 2009) as an outgroup, was refined by eye and analysed in RAxML BlackBox version 8 (Stamatakis et al., 2008; Stamatakis 2014) (GTR model with CAT approximation (all parameters estimated from the data)). A Bayesian consensus tree was constructed using MrBayes v3.2.5 (Ronquist et al., 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for 2 million generations each, with one cold and three heated chains. The evolutionary model applied a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. All parameters were estimated from the data. The trees were sampled every 1000 generations, and the first 500,000 generations discarded as burn-in (trees sampled before the likelihood plots reached stationarity). A consensus tree was constructed from the remaining sample.

Histology and in situ *hybridisation (ISH)*

M. edulis tissues were preserved, prepared for histology and screened for pathogens using light microscopy as in Ward et al (2016). Haplosporidian Type 1-specific probes for ISH were generated by PCR using a sample previously PCR-positive for this sequence type in which haplosporidian plasmodia were visible in histology as template DNA. Type 2-specific probes used a PCR-positive *M. edulis* sample as a DNA template, though no plasmodia were readily visible in the tissue under light microscopy. Probes were labeled using digoxigenin(DIG)-11-dUTP in reactions of 100 µl volume with a final concentration of 1 X Promega colourless buffer, 2.5 mM $MgCl_2$, 20 µM PCR DIG labeling mix (Roche, Germany), 5 U of Promega GoTaq G2 polymerase, 6 µl of template DNA and 0.5 µM each of primers Hap-M258f and Hap-M412r (Type 1) or Hap-E312f and Hap-E620r (Type 2). Cycling conditions were as detailed above for lineage-specific PCR. Tissue sections 4 µm thick were mounted onto Poly-L lysine slides, de-paraffinised by immersion in Clearene (Leica Biosystems), rehydrated and treated with Proteinase K solution (45 µg/ml) for 20 minutes at 37°C in a humid chamber. Proteolysis was terminated by incubation in distilled water and slides rinsed in ice-cold 12% acetic acid for 45 seconds, 70% industrial methylated spirits (IMS) for 3 mins, 100% 3 mins and 2X saline sodium citrate (SSC) buffer for 1 min with agitation. Sections were overlaid with hybridisation solution (4X SSC buffer, 50% formamide, 1X Denhardt's solution, 10% dextran sulphate, 250 µg/ml salmon sperm DNA) containing 10 ng/µl probe (negative controls lacked the DIG-labelled probe in the hybridisation buffer). Slides were heated to 94°C for 7 mins and hybridised overnight at 47°C. After hybridisation, sections were washed in room temperature washing buffer (6M urea, 0.5X SSC and 20 µg/ml BSA), followed

by 2 further washes at 47°C and immersion in 1X SSC at 47°C. Slides were blocked with 6% non-fat milk in Tris buffer (pH 7.5) before incubation with anti-DIG antibodies conjugated with alkaline phosphatase (Roche) in Tris pH 7.5 (1:300) for 1h in darkness. Reactions were developed with 20 µl/ml nitroblue tetrazolium and 5-bromo-4-chloro-3-indoylphosphate (NBT/BCIP) (Roche) in Tris pH 9.5 until staining developed. Sections were then rinsed in Tris pH 9.5 and counterstained with 0.5% Bismarck Brown Y in 30% ethanol, rinsed with tap water and dehydrated in IMS before they were coverslipped with Eukitt (Sigma). Sections were examined under light microscopy for the presence of haplosporidian stages.

Table Ch3-T01. Sequences of primers used in this study for group- and species-specific haplosporidian probing and 18S sequence extension.

Primer name	Primer sequence (5'-3')	Application	Reference
C5fHap	GTA GTC CCA RCY ATA AAC BAT GTC	General haplosporidian 18S nested PCR first round	Hartikainen et al., 2014a
Sb1n	CGA CTT CTC CTT CCT CTA ARY RDT AWG		
V5fHapl	GGA CTC RGG GGG AAG TAT GCT	General haplosporidian 18S nested PCR second round	Hartikainen et al., 2014a
Sb2nHap	CCT TGT TAC GAC TTB TYC TTC CTC		
Hap-M258f	AAC TTT TAG CGT CCA GCC CA	*Minchinia mytili*-specific environmental PCR and *in situ* hybridisation probe generation	This study
Hap-M412r	CGA GGT TGC CAA GTT CTT TCG		
Hap-E312f	CAT AGC AGA TGG AAG TTT GAG G	*Haplosporidium* sp.-specific environmental PCR and *in situ* hybridisation probe generation	
Hap-E620r	GGA GCC AAA TCC GAG GAC TT		
Hap-E449r	TTG GAT GCA CTT TCA AGA TTA CC	*Haplosporidium* sp.-specific 18S reverse primer used for SSU sequence extension	
HapGenFor33	TTG YCT YAA AGA TTA AGC CAT GCA	General haplosporidian 18S forward primer for SSU sequence extension	
HapGenFor84	CTG TGA AAC TGC AKA TGG CTC		

Results

Histology and in situ *hybridisation*

Haplosporidian plasmodia were observed by light microscopy in 6/309 individual *Mytilus edulis* collected in the Tamar estuary. The same haplosporidian sequence type, 'Type 1', was amplified from all six individuals using a nested, haplosporidian-specific PCR. In 5/6 individuals, haplosporidian plasmodia were observed only in the gill and mantle epithelium. In the sixth individual, haplosporidian plasmodia were observed associated with the gonadal tubules in the mantle, gill epithelium, vascular spaces and in the connective interstitial tissues of the digestive gland. In the mantle, unicellular stages were occasionally seen (Fig. Ch3-01A) occurring within the cytoplasm of the tubule epithelial cells. More frequently, larger multinucleate plasmodia containing several cells (up to approximately 12) were observed within the epithelium. In heavily infected tubules the epithelium was disrupted or destroyed. Limited haemocyte infiltration with release of plasmodia into the lumen was seen in a few cases (Fig. Ch3-01B). Evidence of parasite division by cleavage and plasmotomy could be discerned (Fig. Ch3-01A, B & D) and migration of plasmodia through the epithelium of the gonadal tubule into the lumen was also detected (Fig. Ch3-01C). Gill infections were associated with the presence of numerous plasmodial stages within the respiratory epithelium and occasionally in the vascular spaces (Fig. Ch3-01E). No direct evidence of parasite migration through the epithelium was seen. Unlike infections in the gill and mantle, digestive gland infections were associated with mild haemocyte infiltration (Fig. Ch3-01G). TEM examination of digestive gland tissues from infected animals was unable to locate haplosporidian life stages for ultrastructural

characterisation. ISH using the Type 1-specific probe bound to haplosporidian plasmodia in the mantle (Fig. Ch3-01D), gill (Fig. Ch3-01F) and digestive gland (not shown).

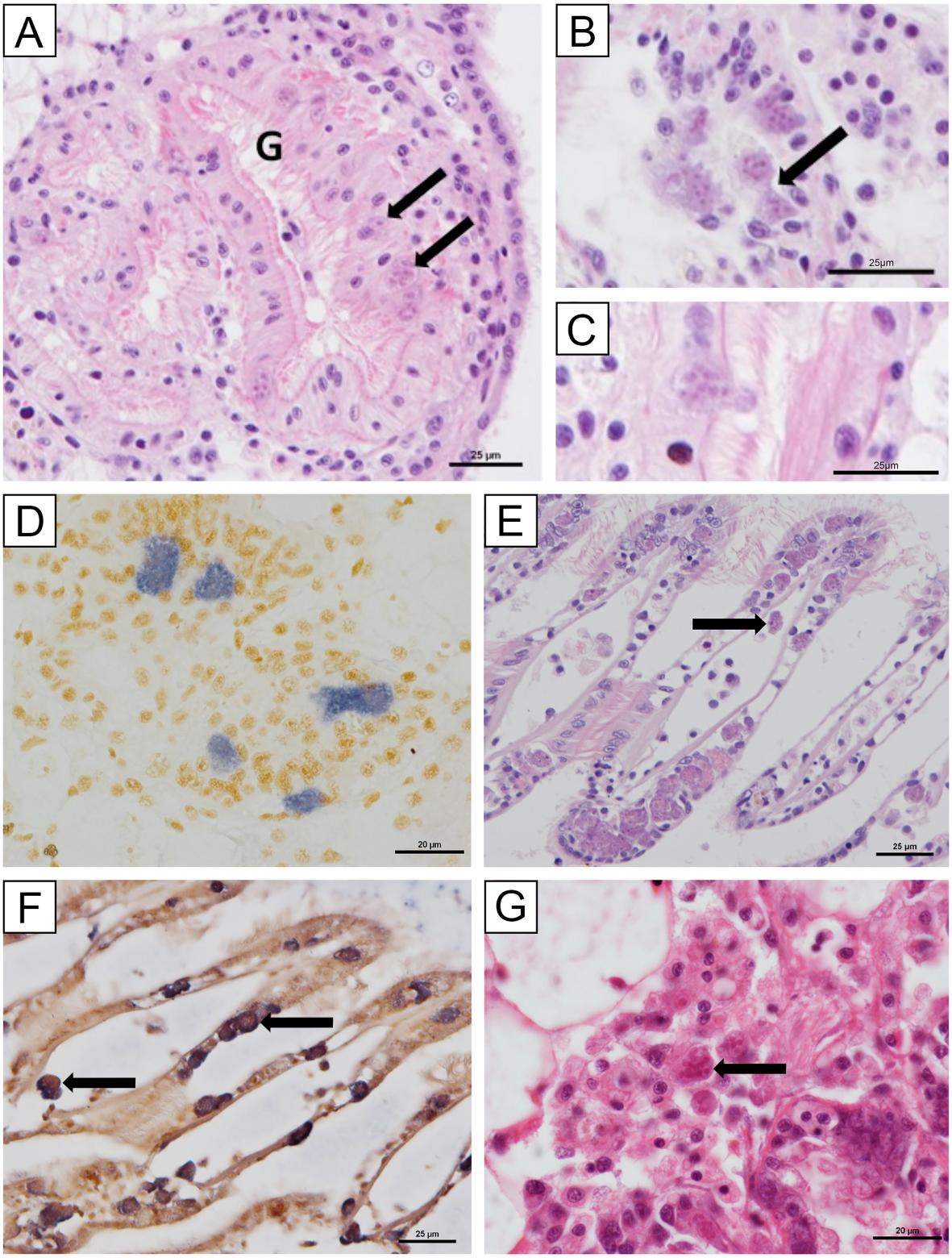

Figure Ch3-01. Gonadal tubes G in the mantle of *Mytilus edulis* harbouring haplosporidian plasmodia. **A.** Section through several tubules, two of which contain unicellular stages and multinucleate intracellular plasmodia (arrows). **B.** Plasmodial division by plasmotomy, each section containing several cells. **C.** Migration of a single plasmodium through the epithelium of the gonadal tubule to the lumen. **D.** Section from the same specimen as (B) showing labelling of plasmodia in a gonadal tubule. **E.** Plasmodia within the gill epithelium with a few also present in the vascular space (arrow). **F**. Same specimen as (E), showing specific labelling of haplosporidian plasmodia (arrow). **G.** Interstitial tissue of the digestive gland with several plasmodia present (arrow).

Of 40 *Mytilus* collected during the investigation of a mortality episode in farmed mussels on the west coast of Scotland, a severe haplosporidian infection was noted in a single specimen (2.5% of sampled population). Numerous plasmodia were observed within the foot muscle, in the gills and throughout the connective tissue surrounding the digestive gland (Fig. Ch3-02). No sporulation stages were observed. Haemocytic infiltration and multifocal granulocytomas were observed in this individual and others sampled. Necrotic cells were present within the granulocytomas. DNA was extracted from formalin-fixed tissue from this individual, and subsequent PCR screens using haplosporidian-specific primer sets amplified a different 18S sequence type than the samples from the Tamar estuary ('Type 2').

A subset of PCR-positive mussels from the Tamar estuary from which the Type 2 sequence was amplified by a single-round (30 cycle) PCR were screened by ISH using a Type 2-specific probe. In each case no staining of plasmodia in any tissues was observed. Haplosporidian cells were also not observed in H&E-stained tissues, and so no microscopic evidence of this haplosporidian infecting *M. edulis* in the Tamar estuary was observed.

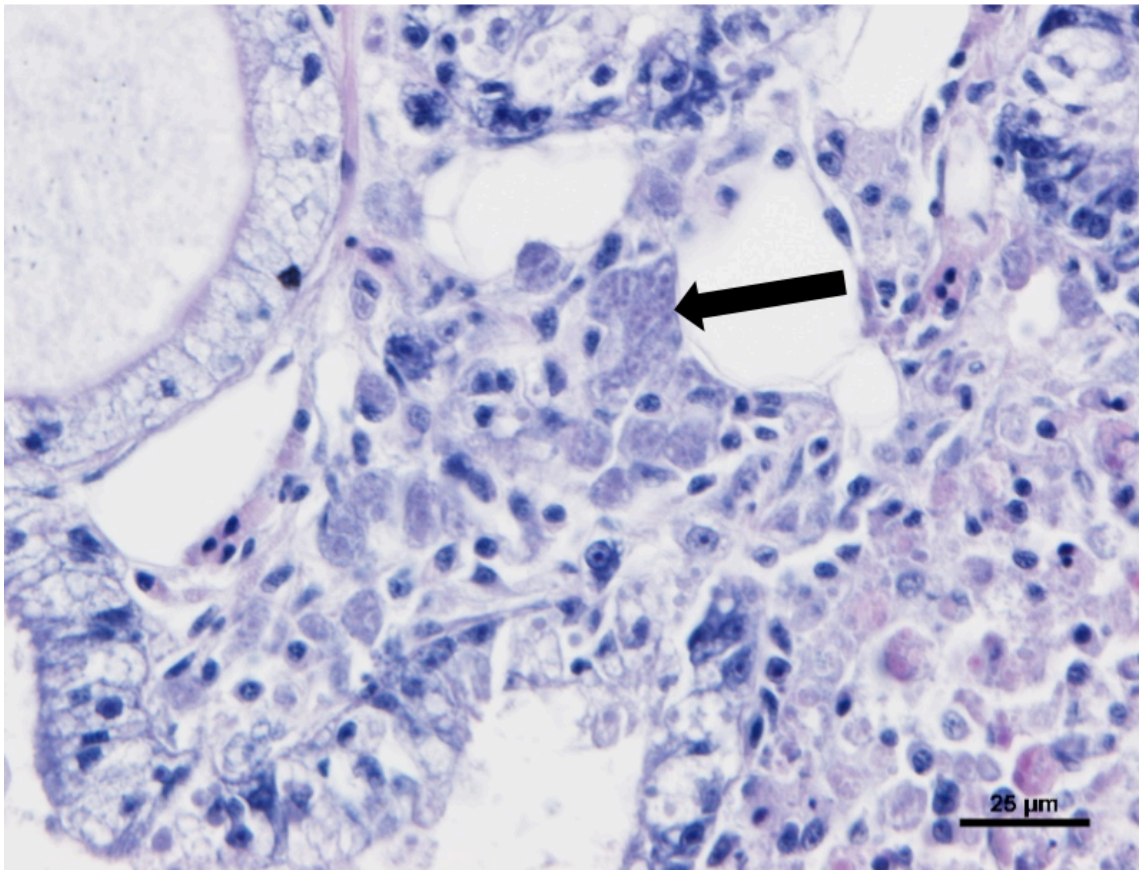

Figure Ch3-02. Interstitial tissue of the digestive gland of *Mytilus edulis* showing several pleomorphic multinucleate haplosporidian plasmodia (arrow).

PCR screening of tissues and environmental samples

A summary of all nested and non-nested PCR screens of tissue and environmental samples is shown in Table Ch3-T02. PCR screens of DNA extracted from the digestive gland tissues of *M. edulis* collected in the River Tamar estuary in June 2013 using general nested haplosporidian primers (Hartikainen et al., 2014a) produced an amplicon in 20/153 samples. Screening of DNA from digestive gland and mantle tissues from *M. edulis* collected at the same site in July 2013 produced amplicons in 63/156 and 12/156 samples respectively. 11/56 mussels sampled produced amplicons from both tissues. Sanger sequencing of amplicons from all mussels produced two distinct haplosporidian sequence types, ‘Type 1’ and ‘Type 2’. Subsequent screens of

the same sample sets using separate 'Type 1' and 'Type 2'-specific primers in a single-round (30 cycle) PCR produced amplicons in 13/309 digestive gland and 7/156 mantle samples for Type 1, and 14/309 digestive gland and 0/156 mantle samples for Type 2. Screening of *M. edulis* incubation filters (16 total; June dataset only) with 'Type'-specific primer pairs resulted in 8 positives from the Type 1 primers when nested with the first round primers of Hartikainen et al. (2014a). and 6 positives when the same primers were used as a single round, 30-cycle PCR. The Type 2 primers produced no positives as either a nested or a single-round PCR. Screens of filtered water samples collected at three sites within the Tamar estuary in June 2013 using the same primer sets and nested and non-nested strategies resulted in amplification from all sites by both primer sets.

Table Ch3-T02. Prevalence of haplosporidian sequence types in *Mytilus edulis* digestive gland and mantle tissues by haplosporidian-targeted nested and specific single-round PCR. Numbers in brackets following sample type indicate the total number of samples screened. For nested PCR, a total of 60 cycles of amplification were used (30 per round); for non-nested PCRs, 30 cycles of amplification were used.

	Minchinia mytili **('Type 1')**		***Haplosporidium*** **sp. ('Type 2')**	
Dataset	**Nested PCR**	**Specific PCR**	**Nested PCR**	**Specific PCR**
June 2013 digestive gland (153)	9	5	11	2
July 2013 digestive gland (156)	22	8	41	12
July 2013 mantle (156)	13	7	2	0
M. edulis incubation filters (June dataset only) (16)	8	6	0	0
Cremyll water column (6)	1	1	6	4
Wilcove water column (14)	5	3	11	6
Neal Point water column (18)	2	0	17	9

Phylogenetic analyses

Phylogenetic analyses including both nearly full-length 18S haplosporidian sequences with all available haplosporidian 18S rRNA sequence types places Type 1 in a strongly supported monophyletic clade comprising all known *Minchinia* spp. plus related environmental sequences (Fig. Ch3-03). As expected from initial BLAST searches of the partial sequence, the closest relative of Type 1 is a previously uncharacterised haplosporidian sequence type derived from mussel tissue collected in the Menai Strait, Wales, UK.

Type 2 falls within a group of uncharacterised sequences from marine water column and tropical oyster gut samples, sister to the oyster parasite *Haplosporidium nelsoni*, with strong support. As in previous studies, branches described as *Haplosporidium* and their relatives do not form a monophyletic clade.

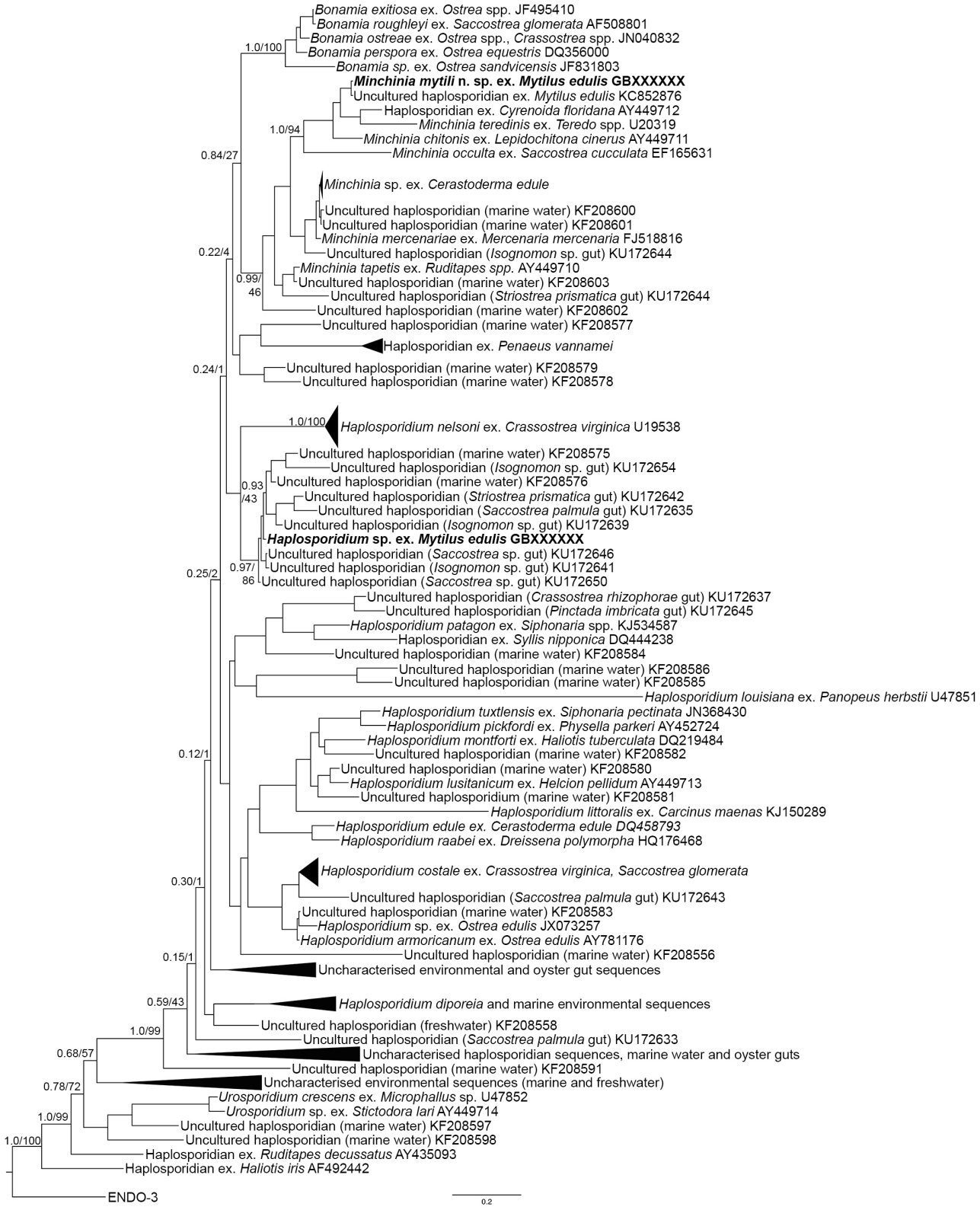

Figure Ch3-03. Bayesian phylogeny of novel haplosporidian sequence types amplified from *Mytilus edulis* tissue. Values on nodes represent Bayesian Posterior Probabilities and Maximum Likelihood bootstrap values respectively.

Taxonomic summary

***Minchinia mytili* n. sp.**

Specific Diagnosis: Plasmodia associated with the gonadal tubes in the mantle, gill epithelium, vascular spaces and in the connective interstitial tissues of the digestive gland. Unicellular stages occasionally present within the cytoplasm of the tubule epithelial cells of the mantle. This species is distinguishable from all other haplosporidians by its unique 18S rRNA gene sequence, which can be specifically amplified by the diagnostic PCR using primers Hap-M258f/Hap-M412r or the *in situ* hybridisation probe generated by them.

Type host: Blue mussel *Mytilus edulis*

Type locality: Cremyll Ferry, Tamar estuary, Devon, United Kingdom (50°21'34.2" N, 4°10'24.9"W)

Site of infection: Systemic, with plasmodia observed associated with the gonadal tubules in the mantle, gill epithelium, vascular spaces and in the connective interstitial tissues of the digestive gland.

Prevalence: Observed in 6/309 individuals examined histologically.

Etymology: The species epithet refers to its infection in the bivalve mollusc host *Mytilus edulis*

Type Material: Reference tissue blocks (histology) and digoxigenin-stained *in situ* hybridisation slides and ethanol-fixed tissue are deposited in the Registry of Aquatic Pathology (RAP), held at the Centre for Environment, Fisheries and Aquaculture Science (Cefas), Weymouth, UK. Reference RA13082-39

Gene Sequence: The 18S rRNA gene sequence has the GenBank Accession Number GBXXXXXX

Zoobank Registration: urn:lsid:zoobank.org:act:XXXXXX

Discussion

This study demonstrates the presence of two novel haplosporidian parasites infecting *Mytilus edulis* in the UK. The first of these, described here as *Minchinia mytili*, was observed infecting the gill, gonadal tubules and digestive connective tissues of mussels in the Tamar estuary, Cornwall, UK. The second, which we informally refer to as *Haplosporidium* sp., was found infecting a single *M. edulis* collected in Loch Spelve, Scotland.

Phylogenetic analyses place the *M. mytili* 18S sequence type within the haplosporidian genus *Minchinia* with strong support, along with other parasites of molluscs including the bivalves *Cyrenoida floridana* (*Minchinia* sp.), *Mercenaria mercenaria* (*M. mercenariae*) and *Saccostrea cucculata* (*M. occulta*). Though spore ornamentation is not available for this species, its infection of a molluscan host and strong phylogenetic placement within the genus is sufficient to describe this novel parasite as *Minchinia mytili*.

The absence of sporogonic stages in all infected individuals examined is unusual for *Minchinia* spp., but it is not without precedent. The hard clam parasite *M. mercenariae* has recently been reported infecting cockles *Cerastoderma edule* in Galicia, Spain, with no observed sporogonic stages (Ramilo et al., 2018). As is true for *M. mytili, M. mercenariae* was present only at very low levels in the sampled cockle population, and so it is possible that in both cases too few infected individuals were available for examination with sufficiently advanced infection.

M. mytili infections were observed in the gill, mantle and digestive gland. Though no direct evidence of parasite migration through the gill epithelium was observed, the presence of haplosporidian plasmodia in the gills may indicate this tissue to be the route of entry into the host, as is the case for other ascetosporean parasites including *Haplosporidium nelsoni* (Lauckner et al., 1983) and *Marteilia* spp. (Kleeman et al., 2002; Carrasco et al., 2008), with the eventual spread of proliferative (plasmodial) stages systemically through the host.

Another *Minchinia* species, *M. occulta,* infects the gill, mantle, reproductive follicles and digestive diverticulae of the rock oyster *Saccostrea cucullata*. Sporulation has been observed only in the connective tissues of the digestive gland (Bearham et al., 2008). Infection in this case is presumed to develop in the gills before disseminating to the mantle and then the digestive gland, which may also be the case for *M. mytili*. Only a single individual was examined where *M. mytili* infection had progressed to the digestive connective tissues. It may be that examination of more infected mussels will reveal sporulation of *M. mytili* within the bivalve host. However, it cannot be ruled out that *M. mytili* does not sporulate in *M. edulis*, but does so outside of this host.

Lynch et al (2014) amplified a haplosporidian sequence type identical to *M. mytili* from the gill tissue of *Mytilus edulis* collected in the Menai Strait, Wales. Though ‘unidentified organisms’ were observed in the tissue of the mussel from which the sequence was amplified, they could not be identified as haplosporidian. However given the presence of the same sequence type in

mussels in the Menai Strait, it is likely the same parasite is present in both populations.

Comps & Tigé (1997) report a haplosporidian producing *Minchinia*-like spores infecting *Mytilus galloprovincialis* in the Thau lagoon, southern France. Infection was limited to the connective tissues surrounding the digestive tubules, with little information to describe plasmodial stages. More recently Matozzo et al. (2018) observed sporocysts containing haplosporidian-like spores in the digestive gland, gonad and mantle of a single specimen of *M. galloprovincialis* collected in Porto Venere, Gulf of La Spezia, Italy. In this case infection was systemic, with necrosis and atrophy of the digestive tubule epithelium associated with granular haemocytes. *In situ* hybridization using a general haplosporidian probe confirmed the affiliation of this parasite with the order. While it is possible both reports are of the same parasite, no sequence data exist in either case to confirm this.

An undescribed haplosporidian observed by Figueras et al (1991) infecting *M. edulis* in Maine, USA shows similarities to *Minchinia mytili*. This parasite was observed infecting the tips of the gills, mantle epithelium and digestive connective tissues. Only plasmodial stages were observed, with no spore stages present. As with other reports of haplosporidian parasites of *Mytilus* spp., no molecular data are available for this parasite, and so it is not possible to state with any certainty their relation to *M. mytili*.

We observe a further novel haplosporidian infecting a single archive specimen of *Mytilus edulis* collected in Loch Spelve, Scotland, UK, after a mortality

episode not attributed to the parasite. Haplosporidian plasmodia were observed in the digestive connective tissue, foot muscle and the gills. Spores were not observed in any tissue. The novel haplosporidian sequence type amplified from this sample, ‘Type 2’, was also amplified from mussel digestive gland tissues from the Tamar estuary, though no haplosporidian plasmodia were observed in any sample by light microscopy or ISH.

Haemocytic infiltration and multifocal granulocytomas were observed in the infected individual and others from the same batch, with necrotic cells present within the granulocytomas. However, scuticociliate, bacterial and other infections were observed within the population (including the haplosporidian-infected individual), which are more likely to be the cause of these pathologies than haplosporidian infection.

Phylogenetic analyses group this parasite with uncharacterised sequence types amplified from water column samples and tropical bivalve guts, sister to oyster parasite *Haplosporidium nelsoni*. This clade is sister to *Bonamia* + *Minchinia*, and so species assigned to the genus *Haplosporidium* are paraphyletic, as in previous studies (Burreson & Reece, 2006; Hartikainen et al., 2014a; Pagenkopp-Lohan et al., 2016).

The haplosporidian infecting this specimen differs greatly from the only *Haplosporidium* species described from mussels, *H. tumefacientis* (Taylor, 1965). Though no sequence data is available for this species, tumefactions observed in the kidney and digestive gland of *Mytilus californianus* infected with

H. tumefacientis are absent the specimen from Loch Spelve, and so they are unlikely to be the same species.

This parasite is distinct from other haplosporidians observed in mussels, and the sequence type associated with the parasite is also novel, however we lack sufficient data to fully describe this species, and so refer to it informally as *Haplosporidium* sp.

The haplosporidian sequence type associated with *Haplosporidium* sp. was amplified from 41/156 of mussels collected in the Tamar estuary in July 2013. Screening the same samples using type-specific primers in a single-round, 30-cycle PCR reduced this number to 12. No haplosporidian life stages were observed in any individual using light microscopy or ISH. Burreson (2008) draws attention to the misuse of PCR assays in molluscan disease diagnosis, and rightly states that PCR-based detection methods must be validated against established microscopic techniques such as histology and in-situ hybridisation. As such we have no irrefutable evidence of *Haplosporidium* sp. infecting *M. edulis* in the Tamar estuary. PCR positives indicate only the presence of *Haplosporidium* sp. DNA, and in this case may indicate passage of parasite cells or DNA through the gut tubules by filter feeding rather than established infection. Barber & Ford (1992) noted the presence of ingested *H. nelsoni* spores by light microscopy in the digestive lumen of *Crassostrea virginica* collected in Delaware Bay, USA (mean frequency 0.5 spores/section). Their presence appeared to be negatively correlated with *H. nelsoni* infection, suggesting they were unable to directly infect the oyster. In the case of *Haplosporidium* sp. a single histology slide was read for each individual, so any

ingested haplosporidian spores may not be readily apparent. PCR assays are more sensitive than light microscopy, and will also detect haplosporidian DNA from digested cells. This highlights the importance of validating PCR data with complementary light microscopy and ISH when inferring infection in bivalves.

Both novel haplosporidian sequence types were amplified from water column samples collected at the sample site. This finding is not unexpected; Hartikainen et al (2014a) detected a wide diversity of haplosporidian sequence types in water and sediment samples, including both characterised and novel lineages. Though haplosporidians have previously been detected in planktonic metazoans by PCR (e.g. *Bonamia ostreae* (Lynch et al., 2007); *H. nelsoni* (Messerman & Bowden, 2016)), no complementary microscopy or ISH was carried out, and so it is not possible to say whether this is representative of actual infection or trophic interactions. Similarly, it remains to be established how long these molecular signals can persist in the water column. *B. ostreae* has been shown to survive at 15°C in laboratory tests for at least one week (Arzul et al., 2009), however the effects of predation and other external environmental factors are as yet untested. Further investigation is needed to understand the significance of eDNA findings, for example determining ribosomal activity of haplosporidian sequence types in environmental samples (and therefore which lineages represent living, active cells), and elucidating the role of planktonic metazoans and other invertebrates in haplosporidian life-cycles.

Chapter Four:

Environmental Sequencing Fills the Gap Between Parasitic Haplosporidians and Free-living Giant Amoebae

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Chapter Description:

In this study, a significant radiation of novel sequence diversity is revealed with ENDO-3 (Paradinida), a clade previously known only from environmental sample types and now known to form a robustly-supported clade comprising *Paradinium* spp. parasites of copepods, and other undescribed parasites of copepods and larger crustaceans including the spot prawn *Pandalus platyceros*. This study combines a targeted primers, an approach which has been used to great success to uncover diversity within other ascetosporean orders, with sequence data mined from Endomyxa-biased and general eukaryote high throughput sequencing datasets to establish the diversity and distribution of basal ascetosporean sequence types in Icelandic bivalve and water samples, coastal water and sediment DNA and cDNA samples collected as part of the BioMarKs Consortium, and littoral water, sediment and invertebrate-associated sample types. As well as uncovering novel diversity within ENDO-3, novel sequence diversity was detected within ENDO-2, a basal clade previously only detected in deep sea samples (and whose inclusion within the Ascetosporea is uncertain), and two novel clades, ENDO-6 and ENDO-8, which group within the phylogenetic gap between the parasitic Ascetosporea and their closest characterised relatives, the free-living amoebae *Gromia* and *Filoreta*.

Author's Contribution:

Georgia Ward optimised the group-specific primer set for ENDO-3/Paradinida, screened all environmental and invertebrate samples by PCR, and prepared all clone libraries. GMW also performed all phylogenetic analyses, and was heavily involved in the drafting of and revisions to this manuscript for publication.

Abstract

Class Ascetosporea (Rhizaria; Endomyxa) comprises many parasites of invertebrates. Within this group, recent group-specific environmental DNA (eDNA) studies have contributed to the establishment of the new order Mikrocytida, a new phylogeny and characterisation of Paramyxida, and illuminated the diversity and distribution of haplosporidians. Here, we use general and lineage-specific PCR primers to investigate the phylogenetic "gap" between haplosporidians and their closest known free-living relatives, the testate amoebae *Gromia* and reticulate amoebae *Filoreta*. Within this gap are *Paradinium* spp. parasites of copepods, which we show to be highly diverse and widely distributed in planktonic and benthic samples. We reveal a robustly supported radiation of parasites, ENDO-3, comprised of *Paradinium* and three further clades (ENDO-3a, ENDO-3b and SPP). A further environmental group, ENDO-2, perhaps comprising several clades, branches between this radiation and the free-living amoebae. Early diverging haplosporidians were also amplified, often associated with bivalves or deep-sea samples. The general primer approach amplified an overlapping set of novel lineages within ENDO-3 and Haplosporida, whereas the group-specific primer strategy, targeted to amplify from the earliest known divergent haplosporidians to *Gromia*, generated greater sequence diversity across part of this phylogenetic range.

Introduction

The retarian subphylum Endomyxa contains two major classes of parasites, which apparently evolved parasitism independently. Phytomyxea, which infect plant, algal, and stramenopile hosts (Neuhauser et al., 2014) are the sister taxon to predatory vampyrellid amoebae (Bass et al., 2009; Berney et al., 2013; Hess et al., 2012), while Ascetosporea, known members of which infect invertebrates, group in a clade also including large testate and reticulose free-living amoebae, and several uncharacterised environmental lineages (Bass et al., 2009).

Ascetosporea comprises five orders: Haplosporida (Hartikainen et al., 2014a), Mikrocytida (Hartikainen et al., 2014b), Paramyxida (Ward et al., 2016), Claustrosporida and Paradinida (Bass et al., 2009). The first three orders include economically significant pathogens of molluscs and crustaceans, including the causative agents of oyster diseases MSX, QX, Aber disease and bonamiosis (*Haplosporidium nelsoni, Marteilia sydneyi, M. refringens* and *Bonamia* spp., respectively), as well as debilitating diseases of crabs (*Paramarteilia canceri* and *Paramikrocytos canceri*) (Feist et al., 2009; Hartikainen et al., 2014a,b; Ward et al., 2016).

Little is known about Claustrosporida and Paradinida, and both orders were erected on the basis of very few characterised specimens. Only two genera have been proposed as paradinids, *Paradinium* and *Atelodinium*, both originally described by Chatton (1920) from the marine copepods *Acartia clausi* and *Paracalanus parvus*, although these paradinid genera were later synonymised

(Chatton and Soyer, 1973). The extensive study by Chatton (1920) focused on dinozoan ("péridinien") parasites generally, but included genera Chatton considered related to but not necessarily belonging to the Dinozoa: Paradinidae (*Paradinium* and *Atelodinium*), Ellobiopsidae (*Ellobiopsis, Staphylocystis, Ellobiocystis* and *Parallobiopsis*) and Blastuloidae (*Neresheimeria* (=*Lohmanella*)). It is now known that *Ellobiopsis* groups phylogenetically within Dinozoa (Gomez et al., 2009), and *Amoebophyra*, which was affiliated with *Neresheimeria* in Blastuloidae by Neresheimer (1904), is a syndinian. Both of these are therefore dinozoan. Chatton (1920) notes characteristics of paradinids that could not only indicate a relationship with syndinians but also identifies many differences between them.

The first sequence data for *Paradinium* was published by Skovgaard & Daugbjerg (2008), showing moderate support for a sister relationship with haplosporidians. 18S rDNA sequences were generated for two *Paradinium* lineages: *P. poucheti* from *Oithona similis* (PaOi01) and an undescribed *Paradinium* sp. from *Euterpina acutifrons* (PaEu41) (i.e., two different copepod host species). Two sequences from parasites of the spot prawn *Pandalus platyceros* (Bower & Meyer 2002) formed a weakly supported clade with *Paradinium* in the Bayesian phylogeny of Skovgaard & Daugbjerg (2008). Bower and Meyer (2002) reported that the spot prawn parasite (SPP) was phylogenetically related to haplosporidians, which is confirmed by Reece et al. (2004).

Resolving the phylogenetic position of parasitic lineages is often complicated by long branches on trees caused by divergent sequences, compounded by low

levels of taxon sampling in groups that are difficult to sample. The lineage sampling of Endomyxa was increased by using group-specific 18S primers in Bass et al. (2009), revealing novel environmental clades (ENDO-2 and -3), clustering in a moderately supported clade with Haplosporida and the giant testate marine amoeba *Gromia* and reticulate amoeba *Filoreta.* Further analyses indicated that *Paradinium* and SPP grouped with environmental clade ENDO-3 (Bass et al., 2009).

Subsequent studies investigating the diversity of Ascetosporea demonstrated that the use of PCR primers designed specifically to divergent groups reveal further novel diversity, for example, of haplosporidians and mikrocytids (Hartikainen et al., 2014a,b) and paramyxids (Ward et al., 2016). These studies also showed that extracting DNA directly from putative hosts of these parasites is a good way of accessing additional diversity, and suggesting host-parasite associations. As ENDO-2/3, *Paradinium* and SPP occupy interesting evolutionary positions between free-living and parasitic lineages, and likely also harbour unknown diversity, we designed primers to amplify from basal haplosporidians (specifically the haplosporidian parasite of *Ruditapes decussatus (*AY435093) to ENDO-2 (DQ504354/EU567273)). We refer to this phylogenetic range as “paradinids and earlier diverging Ascetosporea” (PEDA).

Copepods are the most abundant metazoans in the marine plankton, and indeed on earth, underpinning the marine trophic network (Turner, 2004). Their role as reservoirs and vectors of parasites of larger invertebrates is increasingly recognised (e.g. Arzul et al., 2014), and a longer standing interest in their symbionts has resulted in a body of work which suggests that their protistan

parasites are dominated by dinozoans (Skovgaard & Saiz 2006; Skovgaard et al., 2005, 2007, 2012), and that they are also basibionts for many suctorian ciliates (Gregori et al., 2016). Nonetheless *Paradinium* species have been observed parasitising a number of copepod species, and studies of seasonal occurrence (Chatton & Soyer, 1973; Skovgaard & Saiz, 2006) suggest the parasites may have a high prevalence (up to 35%). Although we cannot say that all lineages related to *Paradinium* are also parasites of copepods, we propose that ascetosporean parasites of crustaceans are much more diverse in terms of lineage richness and ecology than previously recognised.

Materials & Methods

To investigate diversity of PEDA, we used complementary sequence generation methods using four primer strategies on a broad range of sample types: basal ascetosporean-targeted (PEDA) PCR primers generated amplicons from global water and sediment samples and invertebrate tissues. Endomyxan-biased primers were applied to European coastal marine water and sediment samples, and two sets of broadly targeted eukaryote-wide primers were applied to bivalve and associated water column samples from Iceland. The PEDA amplicons were cloned and Sanger sequenced in order to provide longer sequence reads for phylogenetic analyses. Different regions of the 18S rRNA gene were targeted: the eukaryote-wide and endomyxan-biased primers amplified the V4 hypervariable region (recognised as generally the most variable 18S region suitable for phylogenetic interpretation; Stoeck et al., 2010), the V5-V9 regions were amplified by the targeted PEDA primers (determined by availability of sites for primer design and derived by modifying the comparable haplosporidian

primers used by Hartikainen et al., 2014a). An additional eukaryote-wide amplicon (V3) was used in parallel with the eukaryote-wide V4 primers to test their utility for detecting parasites associated with potential hosts.

Sample collection and nucleic acid extraction

Environmental samples

Water and sediment samples were collected from sites in Weymouth, UK (Fleet lagoon, <10-30 ppt salinity; 50°35'N, 2°28'W, and Newton's Cove; 50°34'N, 2°22'W) in June and October 2011 and April 2012, and three sites along the estuary of the River Tamar, UK (Cremyll Ferry, 50.35°N 4.17°W; Wilcove, 50.387°N 4.201°W; Neal Point, 50.443°N 4.204°W) in June 2013, as described in Hartikainen et al. (2014a,b) and Ward et al. (2016). Water samples were similarly collected (omitting the 0.45 μm filtering step) from sites in Sabah, Borneo, Malaysia in December 2011, the Western Cape, South Africa in 2012 and Florida, USA in June 2014 as described in Ward et al (2016). Sediment and water samples were collected from coastal locations near Blanes, Spain (Balearic Sea), Oslo, Norway (Skagerrak, Oslofjorden), Naples, Italy (Tyrrhenian Sea), Varna, Bulgaria (Black Sea) as part of the BioMarKs Consortium (Logares et al., 2014; Massana et al., 2015). The water was then sequentially filtered and DNA and cDNA generated as in Massana et al. (2015). Deep-sea water samples were described in Bass et al. (2007a).

Invertebrate tissue samples

Tissue from abundant invertebrates, including amphipods, mussels, nudibranchs, polychaetes and crabs, was collected from the sites in Weymouth, the Tamar estuary and Florida, and preserved in 100% ethanol as described in

Hartikainen et al. (2014b) and Ward et al. (2016). DNA was extracted from the tissue samples using the DNeasy Blood & Tissue Kit (Qiagen, Germany). Blue mussels (*Mytilus edulis*) and Icelandic scallops (*Chlamys islandica*) were collected together with corresponding seawater samples near the islands of Kiðey and Purkey in Breiðafjörður, West Iceland, in June and August 2010, July and August 2011, and January 2012. Guts were dissected out of the bivalves and their contents collected in 100% ethanol until further processing. DNA from bivalve gut contents and corresponding water samples was isolated using the PowerSoil DNA Isolation Kit (MoBio Laboratories).

PCR, Sequencing, and Sequence Processing

18S rDNA V5-V9 region amplicons

Primers were designed to amplify the V5-V9 region of the SSU gene based on all known sequence data from basal ascetosporean lineages, as of June 2013. The primers were designed to detect diversity between ENDO-2 (DQ504354/EU567273) and the deep-brancing haplosporidian parasite of *Ruditapes decussatus*(AY435093), inclusive of known environmental sequences and crustacean parasites but excluding *Gromia, Filoreta* and most Haplosporida. These primers were applied to water and sediment samples from around the world, and invertebrate tissue from the UK and Florida.

A nested PCR protocol was designed, using primers V4fAsce and SB1n for the first round, followed by V5fAsce and EndoR1 for the final round (Table Ch4-T01). All PCR reactions were conducted in a 20 µl final volumes with 1µl f template DNA and a final concentration of 0.5 µM each primer, 0.4 mM dNTPs, 2.5mM $MgCl_2$, 1X Promega Green Buffer and 0.5 U Promega GoTaq. All PCR reactions were carried out in an ABI Veriti Thermal Cycler. Cycling conditions

for both rounds of the nested protocol consisted of denaturation at 95 °C for 5 min, followed by 30 cycles of 95 °C denaturation for 30 s, annealing at 65 °C for 1 min and an extension step at 72 °C for 1 min, followed by a 10 min final extension at 72 °C, then stored at 4 °C. Amplicons from environmental samples were pooled by sample type and site and purified using polyethylene glycol and ethanol precipitation. Clone libraries were prepared using the StrataGene cloning kit (Agilent Technologies, Santa Clara, CA, USA).

18S rDNA V4 region amplicons

Two different sets of primers amplifying the V4 hypervariable region of the SSU gene were applied to different sample sets. The Icelandic bivalve gut tissue and water samples were amplified with the general eukaryote 3NDF and V4eukR1 primers as described in Bråte et al. (2010). In addition to the taxon-specific sequences, the primers also contained directional GS FLX Titanium primer and key sequences and, in the case of the forward primer, 14 different Multiplex Identifier (MID) sequences to allow barcoding and multiplexing of samples. PCR reactions were performed in triplicate, pooled, cleaned using AMPure magnetic beads (Agencourt) and quantified using the Quant-iT PicoGreen dsDNA Assay Kit (ThermoFisher Scientific) before being pooled in equimolar amounts according to their MIDs for emulsion PCR and pyrosequencing using the GS FLX Titanium chemistry. A whole PicoTitre plate was used for the analysis, separated into eight regions with 14 different samples per primer pair and four different primer pairs used in each region. All methods were used according to the manufacturers' instructions.

Endomyxa-biased V4 amplicons were generated from European sediment and water samples using a cocktail of primers in a nested PCR protocol: first round-

forward primer s6f and reverse pool EndoRmix; second round – forward pool V4fmix and reverse pool s1256Rmix (Table Ch4-T01). Reaction mixtures were of the same composition as used for the V5-V9 PCRs. Cycling conditions for the first round consisted of an initial denaturation at 95 °C for 3 min, followed by 36 cycles of 95 °C denaturation for 30 s, 66 °C annealing for 30 s and a 72 °C extension step for 1 min 30 s. Amplicons were extended at 72 °C for 1 min before storage at 6 °C. For the second round, these conditions were altered to increase the cycle number to 39 and the annealing temperature was increased to 67.5 °C. The forward primers comprised the relevant sequences in Table Ch4-01, the Roche 454 A adaptor, and either one of three three-nucleotide MIDs or no MID. These four bioinformatically sortable conditions were distributed across three half-runs to enable 16 separate libraries to sequences: DNA/cDNA, water column/sediment, in all combinations for the four sampling sites (a, b, c, d).

18S rDNA V3 region amplicons

Amplification of the V3 regions of the SSU gene from Icelandic bivalve gut and water samples were carried out as given in Medinger et al. (2010). The unnamed primers in that publication were designated the names Med454f and Med454r for the forward and reverse primer respectively.

Sequence processing and definition of OTUs

Icelandic bivalve gut tissue and water samples: the 454 amplicons were processed following the 454 Standard Operating Procedure (SOP) for mothur (http://mothur.org/wiki/454_SOP; accessed September 2012) using mothur version 1,27,0 (Schloss et al., 2009, 2011). Quality control parameters were

chosen according to the 454 SOP with a minimum amplicon length of 100bp and using chimera.uchime for chimera detection. Alignment of the amplicons in mothur was done using the SILVA-compatible reference alignment for eukaryotes (http://www.mothur.org/wiki/Silva_reference_files) based on SILVA v102 (Pruesse et al., 2007; Quast et al., 2013). Taxonomic identification of amplicons used the classify.seqs command with default settings on a mothur-compatible dataset of 71787 eukaryotic sequences derived from SILVA release "SSURef 111" as reference (file available on request from the authors). All sequences identified as belonging to Ascetosporea were extracted from the whole dataset for further phylogenetic analyses.

Endomyxa-bised V4 amplicons: the raw sequence files (SFF files) were processed using QIIME v 1.8.0 (Caporaso et al., 2010). The demultiplexing and quality filtering steps were done using default parameters except for minimum read length (150bp instead of 200bp) and maximum primer mismatches (three instead of zero) to allow for wobbles and ambiguous bases in the primers used. Sequences were trimmed to 100bp, then dereplicated and singletons were removed. OTU clustering of the remaining sequences was done with a threshold of 97% sequence similarity using USearch version 9 (Edgar, 2013). Finally, taxonomy was assigned using the BLAST algorithm (Altschul et al., 1990) against the PR2 reference database (release 191, Guillou et al., 2013) and an OTU table created. Based on this OTU table the untrimmed representative sequences for all ascetosporean OTUs were extracted from the remaining dataset after the quality filtering steps. These "full-length" sequences were used for subsequent analyses.

Phylogenetic analyses

Three 18S alignments were produced (V3, V4 and V5-V9) using the sequences generated above aligned with all available basal ascetosporean, haplosporidian, gromiid and reticulosid and closely related environmental 18S sequences from GenBank, identified by blastn searches in January 2016. In each case sequences were aligned using the e-ins-i algorithm on the MAFFT server (Katoh & Standley, 2013), terminal gaps were trimmed, the alignment was refined manually and regions of ambiguous alignment and large indels were removed (masked) by eye. Bootstrapped Maximum Likelihood (ML) trees were then calculated via the Cipres Science Gateway server (Miller et al., 2010) using RaxML BlackBox version 8.2.9 (Stamatakis 2014; Stamatakis et al., 2008) (GTR + CAT; all parameters estimated from the data); bootstrap values were mapped onto the highest likelihood tree obtained. Closely related sequences were then further collapsed into molecularly defined OTUs (MOTUs) using the criterion that > 3 nucleotide differences (including gaps) in any single variable region in the ampicon defined a unique OTU, as used by Hartikainen et al. (2014a) for the analysis of haplosporidian environmental sequence data.
The ML trees were then re-run, and corresponding Bayesian consensus trees were constructed using MrBayes v 3.2.5 (Ronquist et al., 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for 2M generations each with one cold and three heated chains. The evolutionary model applied included a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. All parameters were estimated from the data. Trees were sampled every 1,000 generations. 500,000 generations were discarded as “burn-in” (trees sampled before the likelihood plots reached a plateau) and a consensus tree was constructed from the

returning sample. Sequences are deposited in GenBank (accession numbers MG746635-778).

Table Ch4-T01. Sequences of primers used to generate amplicons covering different regions of the 18S rDNA gene. “Pool ratio” indicates the ratio of primers added to V4 endomyxan-biased reaction pools (final working stock concentration 10 µM)

Gene region, target taxa	Samples screened	Primer Name	Primer Orientation	Primer Sequence (5’-3’)	Pool	Pool Ratio	Reference
V3, general eukaryote	Icelandic bivalve guts, water	MED454f	Forward	ATTAGGGTTCGAATTCCGGAGAGG			Medinger et al. (2010)
		MED454r	Reverse	CTGGAATTACCGCGGSTGCTG			
V4, general eukaryote	Icelandic bivalve guts, water	3NDF	Forward	GGCAAGTCTGGTGCCAG			Bråte et al. (2010)
		V4EukR2	Reverse	ACGGTAATCTRATCGTCTTCG			
V4, Endomyxa-biased eukaryote	European coastal filtered water, sediment; DNA and cDNA	S6f	Forward	GAGGRMAAGYCTGGTGCCAGCASC			
		V4fEuk	Forward	CCAGCASCCGCGGTAAYWCC	V4f Mix	1	
		V4fEnd		GTGCCAGCAGCCGCGGTAAYA		1	
		EndoR0	Reverse	CGACTTCTCCTTCCTCTAAATGATAAG	EndoR mix	1	
		EndoR1		CGACTTCTCCTTCCTCTAARYRDTAWG		1	
		EndoR2		CGACTTCTCCTTCCTCTAARYGHYWWG		1	
		EndoR3		CGACTTYTCCTTCCTCTARATRDYAWG		1	

Gene region, target taxa	Samples screened	Primer Name	Primer Orientation	Primer Sequence (5'-3')	Pool	Pool Ratio	Reference
V4, Endomyxa-biased eukaryote	European coastal filtered water, sediment; DNA and cDNA	C0	Reverse	CACCACCCATAGAATCAAGAAAGATCTTCA	S1256	16	
		4B		CACTAHCCATAGAATCAAGAAAGRKCTKCA	R mix	4	
		Va		CACYAYCCATAGAATCAAGAAAGATCKTCA		2	
		Ph		CACYACCCATAGAATCAAGAAAGAGCTKCA		2	
		Ha		CACYATKCATAGAATCAWGAAAGAACTTBA		2	
		Fi		CACCACCCAYAGAATCAAGAAAGRTCTTCA		2	
		Pl		CACCACCGAAGTGATCAAGAAAGAKCTKCA		1	
		12		CACCAMCCAWAGAATCAAGAAAGATCTGCA		1	
		Re		CACCAMCCATMRAATCAAGAAAGATCTTCA		1	
		Gr		CACCACCCATAWAATCAAGWAAGAKCTKCA		1	
V5-V9, Paradinids and earlier-diverging Ascetospore a (see grey area on Fig. Ch4-01).	Coastal and littoral water, sediment DNA & cDNA; invertebrate tissue and incubations	V4fAsce	Forward	GGAATAATAWGATAGGACTTCRGCA			
		Sb1n	Reverse	GATCCHTCYGGAGGTTCACCTACG			
		V5fAsce	Forward	GYTCRGCACCKTATTYGAGAAATCA			
		EndoR1	Reverse	CGACTTCTCCTTCCTCTAARYRDTAWG			

Results

Analysis of data generated using general eukaryote primers targeting the V3 regions of the SSU gene resulted in 229 or 170,169 (0.13%) sequences belonging to Ascetosporea. Analysis of data generated from the same samples using V4-targeted primers produced 101 of 62, 914 (0.16%) ascetosporean sequences. Between 1 and 6% of sequences generated using Endomyxa-biased V4 primers belonged to Ascetosporea. The group-specific PEDA primers, targeting the V5-V9 regions of the SSU gene, produced only ascetosporean sequence types.

Separate phylogenetic analyses of the V5-V9, V4 and V3 alignments produced three trees (Figs. Ch4-01,02 and 03 respectively). The V5-V9 tree includes OTUs generated by the PEDA primer set from global littoral water, sediment and invertebrate tissue samples, and European coastal sediments (lineages labelled “V5” on Fig. Ch4-01). The PEDA phylogenetic range is also shown on Fig. Ch4-01. The V4 analysis, shown in Fig. Ch4-02, combined data from two primer sets: lineages labelled V4 BIOM, amplified from European coastal sediments and water samples (endomyxan-biased primers) and lineages labelled V4 GEN (Icelandic mussel and scallop gut tissue and associated water samples; general eukaryote primers). Lineages labelled V4 BIOMGEN were amplified by both primer sets. The V3 tree includes OTUs generated from Icelandic mussel and scallop gut tissue and water samples using general eukaryote primers (Fig. Ch4-03). On all three trees, OTUs detected in a single library only are shown in grey.

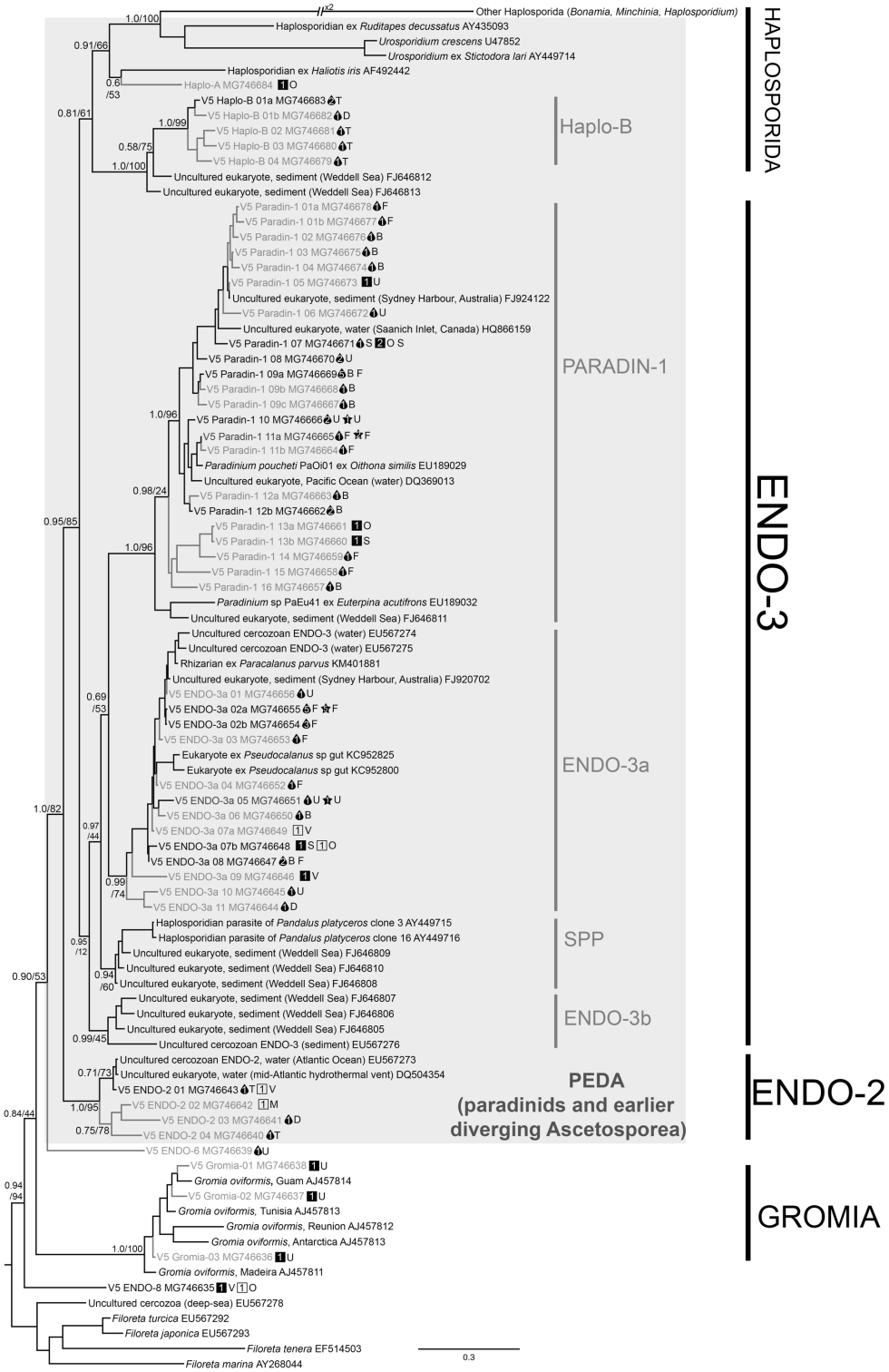

Figure Ch4-01. Bayesian phylogenetic analysis of 18S rDNA V5-V9 region amplicons generated in this study in the context of all available related GenBank sequences, plus representative haplosporidians, *Gromia* and *Filoreta*. The full length of GenBank sequences were used for the analyses. In all figures, values on nodes represent Bayesian Posterior Probabilities and Maximum Likelihood bootstrap values respectively. Numbers in symbols to the right of sequence names show the number of libraries in which each OTU was detected. Squares = sediment (filled = DNA template, open = cDNA template), drop shaped = filtered water, stars = invertebrate tissue and incubation samples. Letters to the right of these indicate (marine) sampling sites. B = Borneo, D = Weddell Sea (deep), F = Florida, USA, M = Mediterranean Sea off Barcelona, Spain, O = Oslofjord, Norway, S = South Africa, T = filtered water near the Titanic wreck, U = UK (South-West), V = Black Sea off Varna, Bulgaria. "ENDO-x" labels of lineages/clades derived from Bass et al. (2009). The shaded area labelled 'PEDA' shows the target region of the V5-V9 primer set devised for this study (see Table Ch4-T01). A larger version of this tree,and Fig. Ch4-02, is included at the end of this thesis.

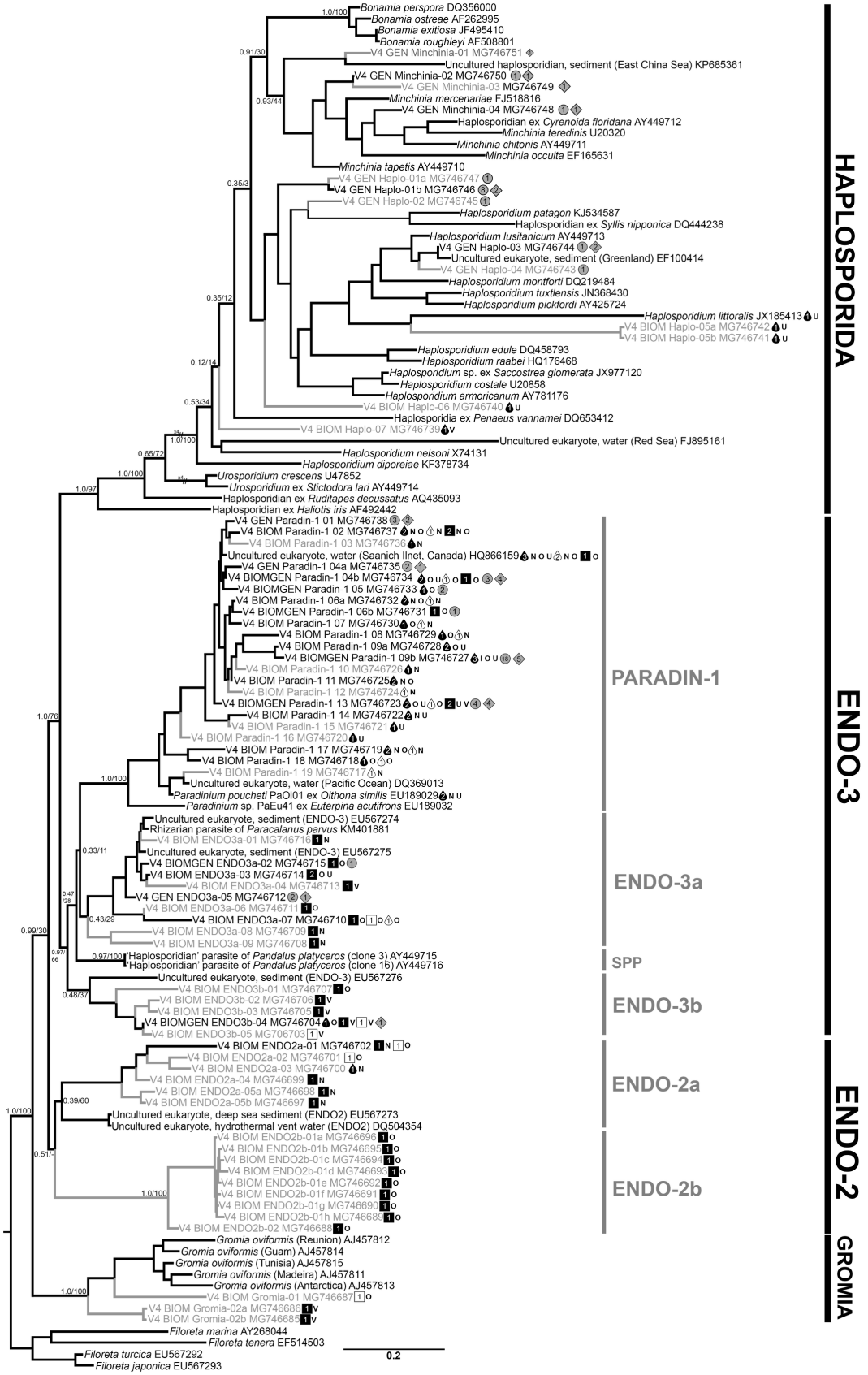

Figure Ch4-02. Bayesian phylogenetic analysis of 18S rDNA V4 region amplicons generated in this study by endomyxan-biased primers and by broadly targeted V4 region primers. All available related GenBank sequences are also included, plus representative haplosporidians, *Gromia* and *Filoreta*. Numbers in symbols to the right of sequence names show the number of libraries in which each OTU was detected. Drop-shaped = filtered water collected at Icelandic bivalve beds, circles = scallop gut tissue, diamonds = mussel gut tissue.

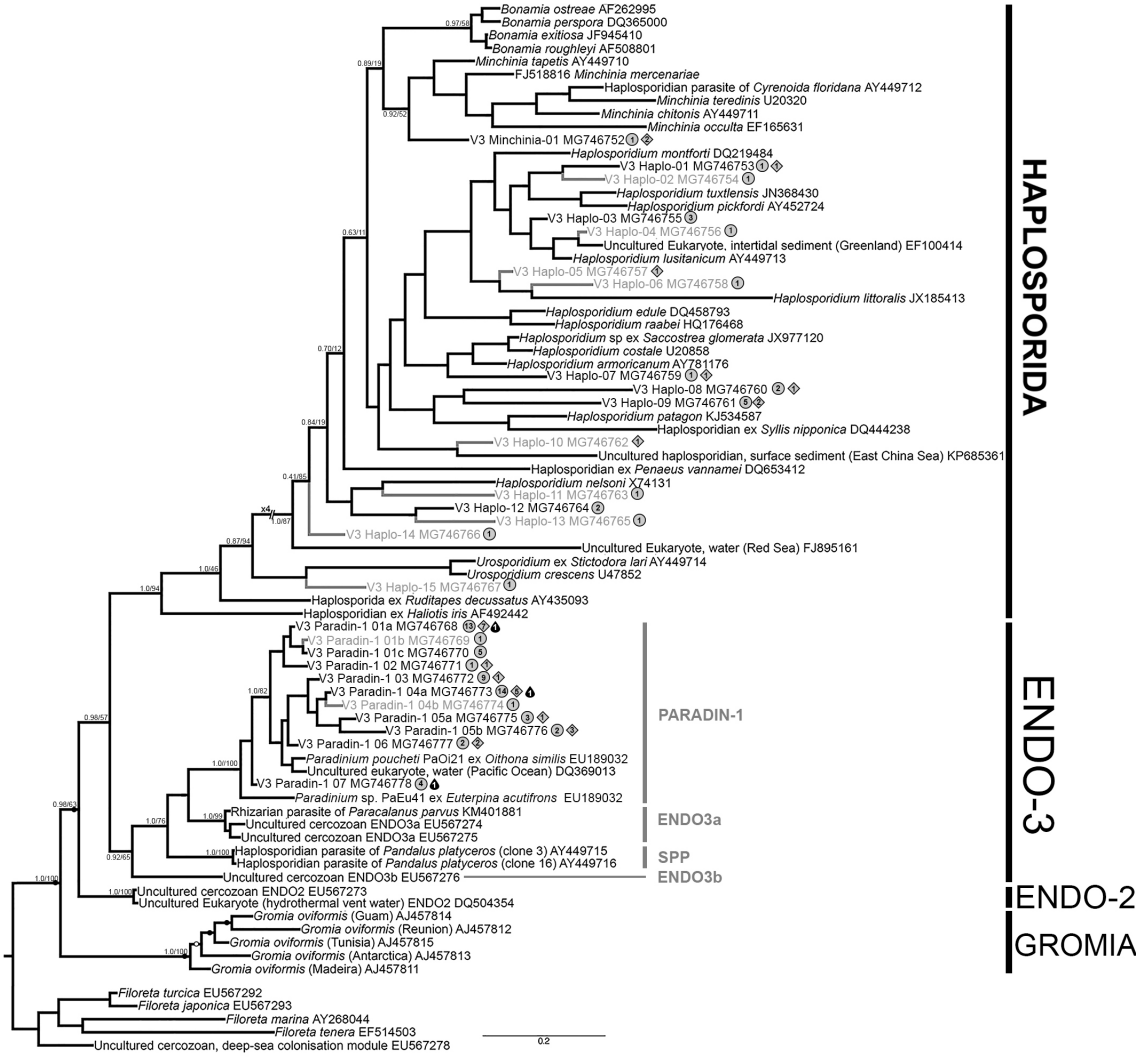

Figure Ch4-03. Bayesian phylogenetic analysis of 18S rDNA V3 region amplicons generated in this study from Icelandic samples in the context of all available related GenBank sequences, plus representative haplosporidians, *Gromia* and *Filoreta*. The full length of GenBank sequences were used for the analyses.

The sequences amplified by different primer/sample strategies group into seven clades, labelled (where present) on each figure as PARADIN-1, ENDO-3a and b, SPP (together forming ENDO-3), ENDO-2a and b, and HAPLO-B. ENDO-3 was sister to Haplosporida in all analyses with moderate support, ENDO-2 being sister to both of them (also moderate support). HAPLO-B is basal to all known haplosporidians.

Other novel sequence types generated outside of ENDO-3 and ENDO-2: (1) the V5-V9 primers (Fig. Ch4-01) amplified a divergent lineage ENDO-6 from a

single littoral water sample from the UK, grouping between PEDA and the closest known relative, *Gromia*. (2) ENDO-8, detected independently from marine sedimnets from Oslo (cDNA) and Varna (DNA), grouping between *Gromia* and *Filoreta*. (3) Three lineages from the Fleet lagoon sediment grouping within the Gromia radiation in the V5-V9 analysis (Fig. Ch4-01), and three further novel sequence types grouping as sisters to *Gromia* (V4 BIOM Gromia-01 and -02), also from Oslo and Varna sediments, in the V4 analysis (Fig. Ch4-02).

Diversity within ENDO-3

PARADIN-1

Sequences belonging to PARADIN-1 were amplified from many sites (Florida, Borneo, UK, Italy, Norway, South Africa) and sample types by all four primer sets. It includes *Paradinium poucheti* (PaOi21) and *Paradinium* sp. (PaEu41) from Skovgaard & Daugbjerg (2008). These are separated by a fundamental bipartition in the clade, with all of the environmental diversity detected in this study belonging to the clade including PaOi21; we detected no other members of the clade including PaEu41. A sediment-derived sequence from the Weddell Sea (FJ646811) groups as sister to this sequence in Fig Ch4-01 (the sequence was omitted from phylogenetic analyses covering the V3 and V4 regions as these are not covered by the sequence). In the *P. poucheti*subclade the majority of lineages detected came from water column DNA samples. None came from BioMarKs coastal sediment cDNA samples. Two lineages closely related to *P. poucheti* (V5 Paradin-1 10 and V5 Paradin-1 11a) were also detected in ascidian tissue (Fleet lagoon) and marine gastropod and oyster incubations (Florida), respectively, as indicated by star annotations on Fig. Ch4-01. No

other sequences in PARADIN-1 derived from invertebrate (-associated) samples in the V5-V9 analysis, however, PARADIN-1 sequence types were commonly amplified from scallop and mussel tissue samples using general eukaryote V4 primers (Fig. Ch4-02).

ENDO-3a, b and SPP clades

ENDO-3 was originally defined in Bass et al. (2009) on the basis of three environmental sequences: EU567274-6. Neither the spot prawn parasite (SPP) nor any confirmed *Paradinium* sequence was included in that paper, therefore, the intergrity of the (moderately well supported) ENDO-3 was not further tested. All of our current trees show that the SPP sequences makes ENDO-3, as originally described, paraphyletic, so we have renamed lineages related to the three above as ENDO-3a (EU567274/5) and b (EU567276). Both ENDO-3a and SPP contain uncharacterised parasites of crustaceans (the copepod *Paracalanus parvus* and prawn *Pandalus platyceros* respectively), whereas the lifestyle of ENDO-3b remains unknown. In the V5-V9 analysis (Fig. Ch4-01), ENDO-3a also contains previously detected sequences from the gut of *Pseudocalanus* spp. copepods (KC952800 and KC952825). We detected novel ENDO-3a lineages not only in water column DNA but also sediment DNA and cDNA. V5 ENDO-3a 02a was detected in crab, sea urchin and zooplankton incubations, all from Florida, and V5 ENDO-3a 05 from an edible mussel incubation (Tamar, UK). Lineages in this clade were detected worldwide, from Florida, UK, the Black Sea, Norway and in the Drake Passage. In the V4 analysis (Fig. Ch4-02), sequences grouping within this clade were found in European coastal sediments and mussel and scallop gut samples.

No sequences generated by any primer set grouped with SPP in any analysis, and ENDO-3b was only detected by the two primer sets used for the V4 analysis. However, the V5-V9 tree is informative as it shows that both SPP relatives and ENDO-3b are present in deep (c. 4,900m) Weddell Sea sediments (Lecroq et al., 2009) (all original ENDO-3 sequences in Bass et al. (2009) were from a range of deep-sea samples).

ENDO-2

ENDO-2 was detected by both V5-V9 and V4 primer sets, from water and sediment samples, DNA and cDNA, but not from any host-associated samples. No sequences within this clade were amplified using the V3 primer set. Although this lineage has been annotated as haplosporidian (DQ504354) on GenBank, all of our phylogenetic analyses show that it is not, and in fact forms a separate clade branching between ENDO-3 and *Gromia*, and so this mislabelling has been omitted from all figures. As is the case for ENDO-3b, there is no morphological evidence for this clade.

ENDO-2 V4 amplicons cluster in three robust but weakly mutually related clades, two of which were unknown prior to this study (ENDO2a and ENDO2b). All sequence types within ENDO-2b were from the same library (Oslo sediment DNA), however, following completion of phylogenetic analyses further BLAST searches of these sequence types against the NCBI GenBank database recovered two environmental sequence types, from Adventfjorden in Norway, showing high sequence identity (98-99%) to V4 BIOM ENDO2b-01a (KT812216) and V4 BIOM ENDO2b-02 (KT810733).

Although true Haplosporida are not the focus of this work it is worth noting that all three primer sets detected diversity in this clade. The broadly targeted V3 and V4 primers amplified a wide range of haplosporidians, which cannot be directly compared to those in Hartikainen et al. (2014a) as amplicons do not overlap. The V5-V9 primer region does overlap but was not targeted to haplosporidians. However, an interesting novel clade, Haplo-B, sister to all other Haplosporida, was amplified from deep sea samples only (from near the wreck of the Titanic), and groups on Fig. Ch4-01 with other deep-sea samples (c. 4,900m) sequenced as part of a study of komoiacean foraminifera in the Weddell Sea (LeCroq et al., 2009).

Discussion

This study is further evidence that PCR primers targeted to defined phylogenetic ranges provide a powerful tool for revealing diversity that more broadly targeted primers either fail to amplify or only produce as a small proportion of large sequence datasets. Here, we designed a primer strategy to investigate the Paradinida, the ascetosporean order for which only a small amount of sequence data exist, and also to populate the region of the ascetosporean phylogeny between the free-living amoebae *Gromia* and *Filoreta* and basal haplosporidians.

We reveal a major novel endomyxan clade, ENDO-3, robustly sister to Haplosporida. Morphological information is available for only two subclades of ENDO-3: two lineages whose morphology is entirely concordant with *Paradinium* (PaEu41 and PaOi01) within PARADIN-1, and the spot prawn

parasite SPP. *Paradinium* has a filo-plasmodial trophic stage which develops into a gonosphere (plasmodial cell mass), from which flagellated dispersal stages are formed. Such plasmodial types and free-swimming zoospores are so far unknown in haplosporids. Other lineages within PARADIN-1 have been detected in planktonic environmental samples and therefore may represent a large radiation of copepod parasites that includes ENDO-3a, although the strongest evidence so far for the latter is their strong planktonic bias and detection in the gut of *Pseudocalanus* spp., and the inclusion within this clade of an uncharacterised parasite of the copepod *Paracalanus parvus*.

Earlier diverging clades within ENDO-3 include SPP, which is the only other lineage between haplosporids and *Gromia* and *Filoreta* for which morphology is known. Similarly to *Paradinium*, SPP does not possess haplosporosomes or lidded spores (as do haplosporids), but SPP differs from *Paradinium* in having unicellular, nonflagellated sessile trophonts developing from undivided plasmodia. Loss of the flagellate condition seems to be common in Endomyxa: the testate amoeba *Gromia* has flagellated gametes but its closest relative, the naked reticulate amoeba *Filoreta* apparently does not. In all of our trees the earliest diverging lineage in ENDO-3 was ENDO-3b, known only from marine benthic samples, some from great depth.

The sister clade to ENDO-3 plus Haplosporida in all analyses is ENDO-2, again only known from benthic or near-benthic habitats, including low oxygen (Varna) and deep-sea samples. Although evolutionary relationships strongly suggest that ENDO-3a is parasitic/symbiotic, and that ENDO-3b might be, the intermediate branching position of ENDO-2 between the free-living amoebae

and ENDO-3 provides less basis for such a hypothesis. The V4 dataset (also the largest in terms of sequence number and sample coverage) also contained ENDO-2b, so far only detected in sediments from Oslo and Svalbard, and the V5-V9 dataset contains ENDO-6, whose phylogenetic position within the Ascetosporea plus Gromia clade is unresolved (Fig. Ch4-01). ENDO-8 may be the closest relative to *Gromia* and *Filoreta* revealed by the study; therefore, we suggest it may resemble those or is a novel amoeboid form. Environmental OTU association analyses (e.g. 'interactome', Science; Lima-Mendez et al., 2015) may suggest potential hosts for ENDO-2 if it is parasitic, but direct evidence is required to prove such an association, for example, via a histological-molecular survey of invertebrates from habitats in which ENDO-2 is known or likely to occur.

While all described paradinid and haplosporids species are parasites of aquatic invertebrates, it should not be asserted that the sequence types generated in this study can definitely stated to be parasites of any particular host, particularly those outside of radiations known to include parasitic lineages (Haplosporida, ENDO-3). The Endomyxan-biased and basal Ascetosporea-targeted primer sets employed focus solely o these lineages, offering no insight into links with potential host groups, and although the 'general' eukaryotic primers may amplify metazoan hosts, only sequence types classified by the bioinformatics pipelines and databases used as Ascetosporea were considered for analyses.

It should also be noted that while sequence types amplified from invertebrate tissue were included in this study, in most cases no complementary samples were collected from these individuals to allow for light microscopy to determine

the presence of absence of ascetosporean life-stages. Particular care should also be taken when interpreting PCR results from bivalve gut tissues, as is the case in this study (see Burreson, 2008), considering their ecological role as filter feeders and increased incidence of environmental contamination when compared to other host and tissue types. Additionally bivalves are not known to be hosts of basal ascetosporean lineages, however both species surveyed from Iceland (*Mytilus edulis* and *Chlamys icelandica*) are known to be parasitised by coepods (Paul, 1983), and such complex relationships such as hyperparasitism cannot be asserted or inferred form the data presented in this study. Future studies should aim to collect specifically-targeted samples of planktonic (and parasitic) copepods, in a manner in which comparable samples can be preserved for both molecular and light microscopy-based technques such as *in situ* hybridisation, to determine the life histories of novel lineages detected in this study as parasites.

The novel deep-branching haplosporids detected (Figs. Ch4-02 and Ch4-03) expand the known ecological range of this order. Many of these were derived from the Icelandic bivalve-associated samples and may represent previously unknown parasites of those bivalves (e.g. V3 Haplo-11, -12 and -13, related to *Haplosporidium nelsoni* (Fig. Ch4-03). V3 Haplo-15 (Fig. Ch4-03) might be a hyperparasite, like its relative *Urosporidium*. The even deeper, exclusively branching, deep-water clade HAPLO-B (Fig. Ch4-01) may represent a radiation of parasites of an unknown (or at least unsampled) bathyphilic invertebrate. Additionally/alternatively some of the Weddell Sea sequences, which were sampled in association with the foraminiferans *Normanina conferta* and *Septuma ocotillo*, may be symbionts ofthose much larger, related protists, in a

similar system to the high protistan diversity recently revealed to be association with radiolarians (sister to foraminifera within the phylum Retaria) (Bråte et al. 2012).

Most known protistan copepod parasites are alveolates (Skovgaard 2014) and euglenozoans (Michalow 1972); this study suggests that Ascetosporea also harbours a large diversity of copepod parasites and has perhaps been more widely overlooked as parasites of other planktonic crustaceans. Certainly their prevalence and diversity in environmental samples merits further investigation. The morphological similarity of paradinid copepod parasites with those elsewhere in the eukaryote tree of life is a further example of striking levels of convergent evolution in protist (and particularly protistan parasite) evolution. An analogous case is the similarity between the cercozoan and stramenopile diatom parasites, *Pseudopirsonia* and *Pirsonia* respectively. Large-scale environmental sequencing studies are revealing massive radiations of lineages for which little morphological information is available, but increasingly, parasites are being characterised within these radiations (e.g. Lima-Mendez et al., 2015), suggesting that much of this newly detected protistan diversity is parasitic. Syndineans and perkinsids are powerful examples of this (Chambouvet et al., 2014, 2015; Guillou et al., 2008), and the diversity revealed in this paper adds to this. We also provide additional evidence that lineage-specific primers are often able to detect higher levels of diversity and/or lineages which are not amplified by broadly targeted 18S primers, and are an important tool for revealing parasite diversity, activity and evolution (Bass et al., 2015; Hartikainen et al., 2014a,b; Ward et al., 2016).

While this study revealed a large number of novel sequence types grouping at the base of Ascetosporea within ENDO-2, -3 and Haplosporida itself, it should be acknowledged that the environmental and organismal samples used during this study were not collected specifically for this work. As such, the manner in which samples were processed and DNA (and RNA) extracted was not optimised for the detection of Ascetosporea, however many of these datasets – particularly the Florida, UK marine sites (Weymouth, Tamar estuary) and Borneo – were collected and processed with a view to screening for closely related ascetosporean orders (Hartikainen et al., 2014a, b; Ward et al., 2016), and the BioMarKs and deep-sea environmental datasets were both collected for studies targeting a broad range of eukaryote taxa, and so were processed as such (Logares et al., 2014; Massana et al., 2015; Bass et al., 2007a). Therefore the somewhat opportunistic use of these sample sets should be considered adequate to provide novel insight into the diversity of basal Ascetosporea, however it is acknowledged that more targeted sampling, particularly the targeting of potential host species and bulk planktonic samples, are important future areas of research for elucidating the diversity within these clades.

Chapter Five:

Haplosporidian Diversity in 'Non-Marine' Environments

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Chapter Description:

Though a number of haplosporidian species have been described from freshwater hosts before, the majority of our knowledge of the diversity and ecology of the group comes from marine environments, and particularly species infecting commercially important hosts. Haplosporidians in marine environments have been shown to infect molluscs, crustaeans and annelid worms, all of which are also found in freshwater environments, though their role as haplosporidian hosts are largely unexplored. This study builds upon previous eDNA studies exploring the diversity of haplosporidians in environmental samples and invertebrate tissues (Hartikainen et al., 2014a; Pagenkopp-Lohan et al., 2016). Hartikainen et al. (2014a) screened a small number of freshwater and terrestrial samples, and detected novel sequence diversity in freshwater filtered water, however the study focused largely on haplosporidians in marine environments and also did not include screens of any invertebrate tissues. In this study, the general haplosporidian primers used by Hartikainen et al. (2014a) are used to screen a much broader range of freshwater environmental samples collected in the UK, Switzerland, Florida and Borneo to explore the diversity and distribution of haplosporidians in freshwater environments. Invertebrate tissues, including potential snail, worm and crustacean hosts (including amphipods, isopods and larger crustaceans) were collected from sites in the UK and Thailand, and screened by PCR to identify potential hosts and novel diversity not detected in water samples. Additionally, terrestrial soils collected from rainforests in Borneo and Peru, and agricultural soils collected in the UK were screened with the same primer set to probe for haplosporidian diversity outside of aquatic environments.

Author's Contribution:

Georgia Ward collected freshwater environmental and invertebrate tissue samples from sites in the UK and performed the majority of DNA extractions and all PCR screens. GMW also designed and optimised the freshwater-targeted haplosporidian primer set used to screen samples for additional diversity, and performed all phylogenetic analyses.

Introduction

Environmental DNA (eDNA) based surveys are an invaluable resource in parasitology, with large-scale sample sets providing the opportunity to screen various non-organismal sample types (such as marine and freshwater sediments and water, and terrestrial soils) for the presence of parasite sequence types without the biases of host-based approaches (Bass et al., 2015). Important outcomes of such surveys include insight into parasite geographic and ecological distribution, as well as life cycle and diversity. eDNA surveys are increasingly being used for the detection of metazoan parasites of importance to human health (e.g. Worrell et al., 2011), and similarly have been used to great effect to increase our knowledge of the diversity and distribution of various parasite groups of significance to aquaculture, such as the Ascetosporea (Hartikainen et al., 2014a,b; Ward et al., 2016; 2018), oomycetes (Holt et al., 2018) and Microsporidia (Ardila-Garcia et al., 2013; Williams et al., 2018). The examples listed here all employed group-specific primer approaches to screen large numbers of environmental samples for the group of interest. This allows for a more targeted approach and is often the only option for extremely divergent parasite groups which are not amplified by the majority of general eukaryote primers. This strategy does however require *a priori* knowledge of sequence diversity within the target group, which may unknowingly lead to amplification biases against divergent or basal members of the group for which sequence data is not yet available.

Primer-based probing of both environmental and invertebrate tissue samples has revealed huge novel diversity within the ascetosporean order Haplosporida (Rhizaria, Endomyxa, Ascetosporea), the majority of which remains

uncharacterised (Hartikainen et al., 2014a; Pagenkopp-Lohan et al., 2016). Though Hartikainen et al. (2014a) screened only a small number of freshwater samples, a number of novel haplosporidian sequence types were amplified, and were distinct from sequence data available for the few characterised freshwater haplosporidian species, *Haplosporidium raabei* (parasite of the zebra mussel *Dreissena polymorpha*) and *Haplosporidium pickfordi* (which infects snails *Physella parkeri*). The main host groups of marine haplosporidians – molluscs, crustaceans and annelid worms – are all found in freshwater and terrestrial environments, though to date few attempts have been made to explore freshwater haplosporidian diversity, and there is scant evidence of haplosporidians infecting non-aquatic hosts. *Haplosporidium meligethi* was described from the rape blossom beetle *Meligethes aenus* (Lipa & Hokkanen, 1991), however few infected specimens were available and no molecular data produced, and the parasite has not been recorded since. Another 'haplosporidian', *Nephridophaga blattellae* was described from electron microscopy infecting the German cockroach *Blatella germanica* (Woolever, 1966), but has since been shown by 18S sequence data to have affinities with the Zygomycota (Fungi) (Wylezich et al., 2004; White et al., 2006).

In this study the nested PCR primer set developed by Hartikainen et al. (2014a) is used to screen a range of freshwater water, sediment and invertebrate tissue samples collected in Europe, Florida and Borneo, and terrestrial soil samples collected in the UK, Peru and Borneo. A second primer set, designed around novel freshwater sequence types was used to probe for further diversity in freshwater sample types.

Materials & Methods

Sample Collection and Processing

References containing sample collection and processing information for previously published environmental datasets are listed in Table Ch5-T01A, and Table Ch5-T01B summarises invertebrate tissue samples screened in this study, for which details are given below.

Freshwater datasets include samples from Borneo, the UK, and Florida. Samples from Borneo and the UK were included in the study of Hartikainen et al. (2014a), focusing on haplosporidian sequence diversity in marine environmental samples, and so may allow for limited geographic comparison.

During sampling at UK sites, water was collected from river and lake sites in the New Forest. At these same sites and additional locations in Leeds and Hampstead Heath, London, known haplosporidian host groups, including amphipods, larger crustaceans, snails and annelid worms.

Other samples were available for use in this study, though not collected specifically for this work. These include soil samples, which were included as many potential terrestrial hosts groups for haplosporidians are found in soil, including amphipods and worms. Tissue and incubation samples from freshwater crab species from Thailand and the UK were also included, as marine crabs are known to be hosts of haplosporidian species.

Amphipods *Gammarus pulex* collected from freshwater streams in Leeds, UK were collected in April 2014, and preserved in 70% molecular-grade. DNA was extracted using a standard phenol:chloroform protocol (Nishiguchi et al., 2002). Invertebrates including amphipods, snails, worms and isopods were collected at freshwater sites in the UK (California Lake, Surrey; Bickton and Whirlpool, Hampshire) at the same time as large volume (20 L) water samples were collected and processed as described in Hartikainen et al. (2014b). Invertebrates were preserved in 100% ethanol upon collection, and DNA extracted from invertebrate tissues using the DNeasy Blood & Tissue Kit (Qiagen). Isopods *Asellus* sp. were collected from the river Itchen in June 2014 and preserved in 100% ethanol before DNA was extracted using the DNEasy Blood & Tissue kit.

14 swamp crayfish *Procambarus clarkii* were collected in unbaited traps in the Bird Sanctuary Pond, Hampstead Heath, NW London, UK in April 2016. Animals were kept in aerated tanks of sterile Prescott's and James's Solution (Culture Collection of Algae and Protozoa; www.ccap.ac.uk) at 14°C for 24 to purge, before transfer to tanks containing 20 L fresh solution, where they were incubated with aeration for 42 hours. After incubation, all 20 L of water was filtered under pressure through a 0.45 µm cellulose acetate filter, and filtrand collected and preserved at -80°C prior to DNA extraction using the MoBio PowerSoil kit (MoBio Laboratiories, USA). After incubation animals were anaesthetised by exposure to chloroform and dissected. Samples of gill, gut, gonad, muscle, hepatopancreas and, where visible, antennal gland were preserved in Davidson's Freshwater Fixative for histology and 100% molecular

grade ethanol for DNA extraction. DNA was extracted using the DNEasy Blood & Tissue kit.

Juvenile mitten crabs *Eriochier sinensis* were collected from the River Thames at Chelsea Harbour in May 2014 and incubated in pools of 2-5 individuals in 500 mL Prescott's and James's solution for 150 minutes. After this time, 100 mL of water was filtered through a GF/F filter (Whatmann; GE Healthcare, USA) and preserved at -80 ºC. The crabs were transferred to fresh solution and incubated for a further 24 hours. 100 mL was filtered through a second GF/F filter. DNA was extracted from the filters using the MiBio PowerSoil kit.

Unidentified freshwater crabs were collected from sites in Vietnam and Thailand, and several animals of the same species dissected into pools of muscle, gill and internal tissues, preserved in 70% ethanol. These samples were provided by Andy Shinn (FishVet Group, Bangkok, Thailand). For each species, 8 DNA extractions were performed for each tissue type using the Qiagen Blood & Tissue kit.

Table Ch5-T01A. Previously published datasets used in this chapter.

Sample Code	Dataset	Reference
BOR	Borneo freshwater and terrestrial eDNA	Hartikainen et al., 2014a
FLA	Florida freshwater eDNA, invertebrate incubations and invertebrate tissues	Ward et al., 2016
SWI	Switzerland filtered river water	Hartikainen et al., in prep
UKFW	UK: lake (CAL) & river (BIC, WPL) water	Hartikainen et al., 2014b
EXE	Dartmoor soils and leaf litter	Hartikainen et al., 2014a
PER	Peru soils	Bass et al., 2007a
UKAG	UK agricultural soils	Gosling et al., 2017

Table Ch5-T01B. Invertebrate tissue samples screened in this chapter.

Sample Code	Organism	Sampling Location
UKFW	Amphipods *Gammarus pulex* (n = 35)	Leeds, UK
	Amphipods *Gammarus pulex* (n = 55); Isopods *Asellus* sp. (n = 20); Ramshorn snails *Anisus* sp.(n = 17); Freshwater snails *Physella* sp. (n = 18); Hemiptera sp. (n = 13); *Tubifex* sp. (n = 13).	California Lake (Surrey); Bickton (River Avon)
	Isopods *Asellus* sp. (n = 8)	River Itchen, UK
	Crayfish *Procambarus clarkii* (n = 14)	Bird Sanctuary Pond, Hampstead Heath, UK
	Mitten crab *Eriochier sinensis* incubations (n = 6)	River Thames (Chelsea Harbour), UK
THA	Gill tissue (n = 8), muscle tissue (n= 8) and internal structures (n=8) from two unidentified crab spp.	Vietnam and Thailand

PCR and sequencing

Table Ch5-T02 lists primers used in this study, their specificity and, where applicable, original publication. Haplosporidian-targeted nested primers developed by Hartikainen et al. (2014a) were used to freshwater and terrestrial environmental and invertebrate samples. Resultant amplicons from eDNA were pooled by sample type and site, and clone libraries prepared using the Stratagene cloning kit (Agilent Technologies, Santa Clara, CA, USA), and 12 colonies from each library Sanger sequenced unidirectionally using the M13 primer. Preliminary phylogenetic analyses using sequence types from freshwater environmental samples revealed significant novel haplosporidian diversity, and so separate primer set was designed and used to re-screen freshwater eDNA and invertebrate samples in order to detect additional freshwater diversity. Reactions used forward primer HaploFwF1 and reverse primers HaploFwR1a and HaploFwR1b (pooled equimolarly to a working stock of 10 μM concentration). Reaction mixtures followed the same composition as Ward et al. (2016), and cycling conditions were as follows: initial denaturation at 95 °C for 5 minutes, followed by 40 cycles of 95 °C for 1 min, primer annealing at 58 °C for 45 s and elongation at 72 °C for 45 s, and a final extension step at 72 °C for 10 minutes. Amplicons from environmental samples were pooled according to sample type, size fraction and site before cloning as above.

All invertebrate and terrestrial amplicons were Sanger sequenced unidirectionally (without the need for cloning) using the forward primer (either V5fHapl or HaploFwF1).

Phylogenetic analyses

All available full-length 18S haplosporidian sequence types, including uncharacterised lineages identified by blastn searches of the NCBI GenBank database in August 2018, were aligned with novel sequence data generated in this study using MAFFT version 7 (e-ins-i algorithm) (Katoh & Standley, 2013). The alignment was refined by eye and analysed using Maximum Likelihood (ML) in RaxML BlackBox version 8 (Stamatakis, 2014) (Generalised time-reversible (GTR) model with CAT approximation (all parameters estimated from the data); an average of 10,000 bootstrap values was mapped onto the tree with the highest likelihood value).

Table Ch5-T02. Primers used to generate haplosporidian sequence types from freshwater and terrestrial environmental and invertebrate tissue samples.

Primer	**Specificity**	**Reference**
C5fHap: GTA GTC CCA RCY ATA AAC BAT GTC S2nHap: CCT TGT TAC GAC TTB TYC TTC CTC V5fHapl: GGA CTC RGG GGG AAG TAT GCT Sb1n: GAT CCH TCY GCA GGT TCA CCT ACG	General Haplosporida (including marine and freshwater lineages), 18S V5-V9	Hartikainen et al., 2014a
HaploFWf1: GAC CTC AGC CAT CTA AYT AGC HaploFWr1a: CCA CTC AAT TCA CCG GAT TAT TC HaploFWr1b: CTA TCC ACT TAA TTC ACT GTG TTA TTC	Freshwater Haplosporida, 18S V6-V8	This study

Results

The results of PCR screens of environmental and invertebrate tissue samples using the general haplosporidian primer set of Hartikainen et al. (2014a) and the freshwater haplosporidian primer set developed for this study are shown in Table Ch5-T03, which also includes the results of previously-published screens of freshwater and terrestrial samples by Hartikainen et al. (2014a) (shaded in grey), which were included in phylogenetic analyses. Both primer sets amplified novel sequence diversity in freshwater filtered water and invertebrate tissue samples, however only the 'general' primers detected haplosporidian sequence types in terrestrial soils. The freshwater primers amplified haplosporidian sequences from all freshwater sample types screened, and produced amplicons from a much greater number of samples in most cases. The only exception is river water samples collected on the River Wigger and its tributaries in Switzerland: though a greater number of samples amplified using the 'general' primer set (56/77, compared with 41/77 using the freshwater primers), sequence types amplified from these samples fall largely within the core *Haplosporidium* and 'Clade B' (see Fig. Ch5-01), which also includes novel terrestrial lineages amplified from soil (also from the 'general' primers only), and did not amplify any sequence types in Clade C or any of the five novel lineages (see below).

The freshwater primers also produced haplosporidian amplicons from a significantly higher number of water samples collected in Borneo (51/94 compared to 4/94 using the 'general' primers) and Florida (16/34 compared with 1/34). This primer set also detected haplosporidians in a greater number of invertebrate tissue samples, particularly amphipods (12/55 compared to 3/55),

Ramshorn snails (12/17 compared with 9/17) and *Tubifex* worms (9/13 compared with 2/13) collected at river and lake sites in southern England.

Table Ch5-T03. Results of PCR screens of freshwater water and invertebrate tissue samples and terrestrial soils using 'general' haplosporidian-targeted (Hartikainen et al., 2014a) and freshwater haplosporidian-targeted primers. Rows shaded grey indicate results previously published in Hartikainen et al. (2014a). NS indicates samples were not screened using that primer set.

Sampling Location (Sample Code)	Sample Type	Primer Set (no. positives/no. screened) 'General' haplosporidian	Freshwater haplosporidian
South Africa (SA)	River water	8/54	NS
	River sediment	2/9	NS
Panama (PAN)	River water	0/8	NS
	Terrestrial soils	0/3	NS
Borneo, Malaysia (BOR)	River and pond water	4/94	51/94
	Rainforest soils	2/8	0/8
Florida (FLA)	River and lake water	1/34	16/34
	Amphipod tissue	1/29	5/29
River Wigger, Switzerland (SWI)	River water	56/77	41/77
California Lake, Surrey; Bickon, River Avon (UKFW)	River and lake water	31/80	34/80
	Amphipod *Gammarus* tissue	3/55	12/55
	Isopod *Asellus* tissue	1/20	3/20
	Ramshorn (*Anisus)* tissue	9/17	12/17
	Snail (*Physella*) tissue	4/18	6/18
	Hempitera tissue	1/13	5/13
	Worm *Tubifex* tissue	2/13	9/13
Hampstead Heath Pond (UKFW)	Crayfish *Procambarus* tissue	2/14	NS

Sampling Location (Sample Code)	Sample Type	Primer Set (no. positives/no. screened)	
		'General' haplosporidian	Freshwater haplosporidian
Leeds (UKFW)	Amphipod *Gammarus* tissue	NS	10/35
Itchen (UKFW)	Isopod *Asellus*	0/8	4/8
River Thames (UKFW)	Crab *Eriochier* incubation filters	2/8	NS
Thai crabs (THA)	Thai crab tissues	4/48	NS (true?)
Dartmoor (EXE)	Leaf litter	0/8	0/8
UK (UKAG)	Agricultural soils	7/22	0/22
Peru	Rainforest soils	2/8	0/8

As shown in Fig. Ch5-01, freshwater and terrestrial diversity detected in this study groups into five novel lineages, with significant diversity also detected within Clade B and Clade C of Hartikainen et al. (2014a). Additionally, three novel sequence types were also detected within the "core" *Haplosporidium*, which includes described freshwater species *H. pickfordi* (parasite of *Physella* spp. snails) and *H. raabei* (parasite of the zebra mussel *Dreissena polymorpha*). No novel sequence diversity was detected within the genera *Bonamia, Minchinia* or *Urosporidium*. Though most clades receive moderate or strong Bootstrap support, support for the overall phylogeny is very low.

Novel lineage 1 was amplified from lake water collected in Surrey, UK and Lake Kerr, Florida. Sequences within this lineage were also amplified from amphipods collected in the new forest (4/55), and from single specimens of *Tubifex* worms and Ramshorn snails (*Anisus* sp.) collected at the same site.

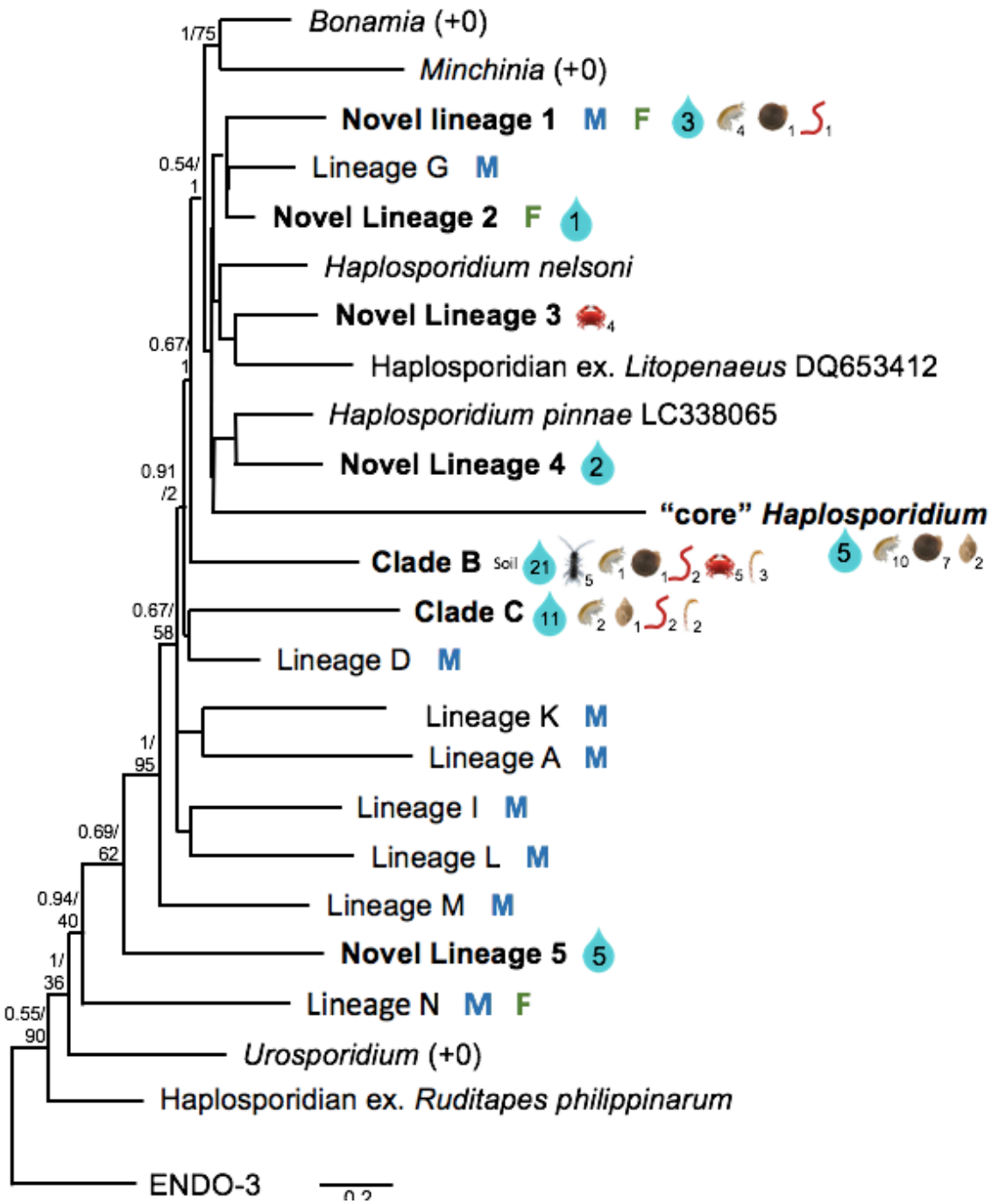

Figure Ch5-01. Maximum Likelihood phylogeny of haplosporidian sequence types amplified from freshwater and terrestrial environmental samples and freshwater invertebrates, with uncharacterised sequence types from marine environmental samples and all full-length haplosporidian sequence types from GenBank. Values on nodes represent Bayesian posterior probability and Maximum Likelihood Bootstrap support respectively. Branch labels annotated with a blue M are lineages detected in marine environments, and those with a green F those amplified from freshwater sample types. Numbers associated with droplet and invertebrate annotations indicate the number of samples from which the lineage was amplified.

Novel Lineage 2 was detected in a single river water sample collected in the New Forest, UK. This lineage was not detected in any invertebrate sample collected from the same site.

Novel Lineage 3 was the only novel lineage not to be detected in environmental samples, and was amplified from the tissues of two unidentified crab species. The same sequence type was amplified from the gills, muscle and internal tissues of a small, unidentified crab collected in rivers in Chonburi Province, Thailand, and the muscle tissue of a different unknown species collected near a waterfall in Rayong Province, Thailand.

Novel Lineage 4 was detected only in Borneo, in water samples collected from the Agathis River, and a manmade pond in the Maliau Basin, and Novel Lineage 5 from the same sites, plus lake water collected in Surrey, UK.

The 'Core *Haplosporidium'* clade, first described as such by Hartikainen et al. (2014a) is a poorly-supported clade comprising nearly all described *Haplosporidium* species for which 18S sequence data is available (with the exception of *H. nelsoni, H. diporeiae* and *H. pinnae*). Inclusion of other *Haplosporidium* spp. within this definition would result in paraphyly of the genus, and so until sequence data is available for the *Haplosporidium* type species, *H. scolopi*, or a neotype is designated, it remains uncertain whether this clade contains true *Haplosporidium* spp.

Within this clade, three novel sequence types were amplified. The first of these, sister to *Haplosporidium pickfordii*, was amplified from a single amphipod

collected in the St John's River in Florida, USA. A second novel sequence type, detected in water samples collected on the River Avon in the New Forest, UK and the River Wigger, Switzerland, was also amplified from 7/17 Ramshorn snails *Anisus* sp. collected in the river Avon. The third novel sequence type groups at the base of core *Haplosporidium*, and was found in water collected from rivers in the UK and Switzerland, and 9 of 34 amphipods collected from a lake near Leeds, UK, a single Ramshorn snail collected in the New Forest, and a single *Physella* sp. snail collected at the same site.

Haplosporidian Clade B was originally detected by Hartikainen et al. (2014a) in a water sample collected from a bog on Dartmoor, Devon, UK. All sequence types amplified from terrestrial samples (rainforest soils collected in Borneo and Peru, and agricultural soils collected in the UK) group within this clade. Few invertebrate-derived sequences fall within this radiation, though novel sequence types were amplified from mitten crab *Eriochier* incubations and tissues of crayfish *Procambarus clarkii*. Histological examination detected no haplosporidian life-stages in any crayfish tissue. Sequence types within Clade B were also amplified from river, lake and pond water samples collected in the UK, Florida and Borneo. Phylogenetic analysis shows that Lineage F of Hartikainen et al. (2014a), amplified from brackish sediments collected in the Fleet Lagoon, Dorset, also groups within this clade, though support is low (Fig. Ch5-02).

Haplosporidian Clade C was largely amplified from rockpool water samples by Hartikainen et al. (2014a). Since the publication of this study sequence data has become available for *Haplosporidium diporeiae*, parasite of the freshwater

amphipod *Diporeia* spp. in the Laurentian Great Lakes, USA (Winters & Faisal., 2014), which also groups within this clade (see Fig. Ch5-02). Novel sequence types in this clade are distinct from marine sequence types, with the exception of 'Clade C_07', amplified from freshwater *Tubifex* collected from a river in the New Forest, which is identical to HAPLO_18 of Hartikainen et al. (2014a), first detected in Newton's Cove, Weymouth, UK. Most sequence types within this clade were amplified from samples collected at UK freshwater sites, except 'Clade C 05', detected in water samples from 4 sites in Borneo and 3 sites in Florida as well as 3 sites within the UK, and Clade C 08, amplified from river water collected in the New Forest, UK, and Florida, USA. No sequence types within this clade were amplified from terrestrial soil samples or river water samples collected along the River Wigger and its tributaries in Switzerland.

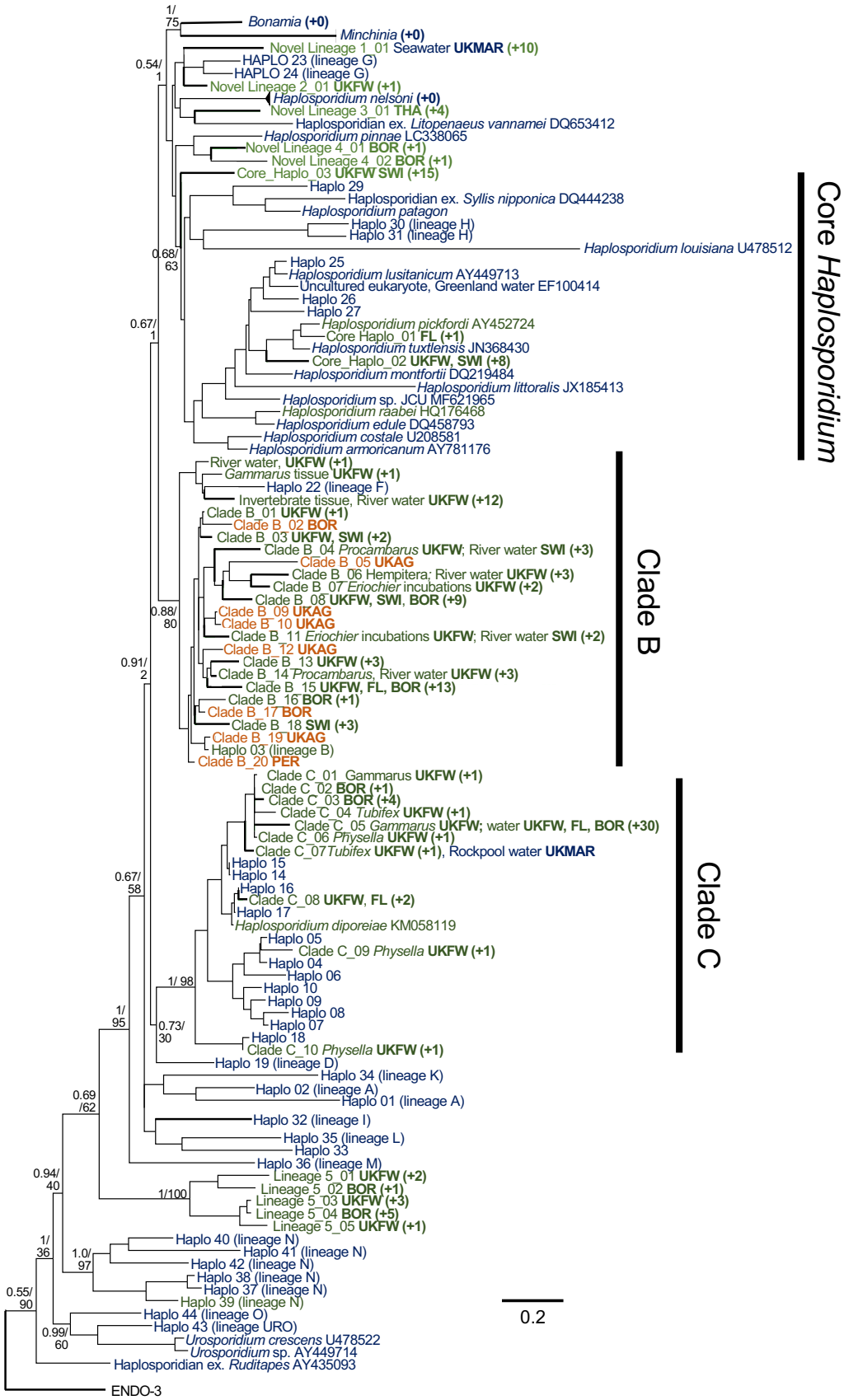

Figure Ch5-02. Maximum Likelihood phylogeny of all available overlapping haplosporidian sequence data with newly-generated freshwater and terrestrial sequence types. Branch labels in blue indicate sequence types found only in marine environments, green those in freshwater environments and orange those in terrestrial soils. Values on key nodes indicate Bayesian posterior probability and Maximum Likelihood bootstrap values respectively.

Discussion

Figure Ch5-01 shows the phylogenetic position of the five novel lineages amplified from freshwater environmental and invertebrate tissue samples, and Clade B and Clade C of Hartikainen et al. (2014a), into which a significant proportion of novel diversity falls. No novel diversity groups within the genera *Bonamia, Minchinia* or *Urosporidium*. Both *Bonamia* and *Minchinia* are known entirely as parasites of marine molluscs, particularly bivalves, and so it is not surprising that no novel diversity within these genera was amplified, as no freshwater bivalves were collected as part of this study. Characterised *Urosporidium* species are parasites of marine free-living annelids and trematode worms, whose freshwater and terrestrial counterparts were similarly undersampled.

Three novel sequence types grouped within a clade containing the majority of characterised *Haplosporidium* species for which sequence data are available (Fig. Ch5-02, labelled core *Haplosporidium*), including two freshwater *Haplosporidium* species, *H. pickfordii* and *H. raabei*. Of these, one sequence type, 'Core Haplo 02', was amplified from 7/17 (41%) of Ramshorn snails *Anisus* sp. collected from the River Avon in the New Forest, UK. *H. pickfordi*, a close relative of this lineage, is a parasite of freshwater snails, and so further sampling, with a focus on collecting complementary material for molecular and microscopic examination, is warranted. The third sequence type within this clade, 'Core Haplo 03', was amplified from 9/55 *Gammarus* sp. amphipods collected on the River Avon, UK and lakes in Surrey, UK (Fig. Ch5-01, -02).

Haplosporidium diporeiae, recently described from freshwater amphipod hosts *Diporeia* spp., groups within Clade C of Hartikainen et al. (2014a), as shown in Fig. Ch5-02. Sequences within this clade have been linked to haplosporidian parasites of amphipods in littoral environments (Urrutia et al., in prep), and as-yet uncharacterised (and unsequenced) haplosporidians morphologically distinct from *H. diporeiae* have been observed infecting the same amphipod hosts in the Great Lakes, USA (Winters & Faisal, 2014; Messick et al., 2004; Messick, 2009), suggesting freshwater amphipods may be hosts of several haplosporidian species.

Haplosporidium gammari, parasite of the freshwater amphipod *Gammarus (Rivulogammarus) pulex*, was reassigned to the family Claustrosporidiidae and genus *Claustrosporidium* by Larsson (1987) on the basis of morphological characters observed in a single fixed specimen. One of only two members of the ascetosporean order Claustrosporida Cavalier-Smith 2003, *C. gammari* is still known only from this single specimen and no sequence data are available. Greater sampling effort of freshwater *Gammarus* amphipods, including the collection of material for molecular work and light and electron microscopy, may provide the necessary evidence to validate the existence of the order Claustrosporida, and elucidate its relationships with Haplosporida. It is also possible that *C. gammari* is a morphologically divergent haplosporidian, grouping within one of the uncharacterised clades from this study, however this cannot be determined until complementary morphological and molecular data are available for a larger number of ascetosporean parasites of freshwater amphipods. The second member of the genus *Claustrosporidium, C. asellii*, was described infecting *Asellus* sp. isopods, and was also originally described within

the genus *Haplosporidium* (Larsson 1987). A novel sequence type, sister to marine lineage F of Hartikainen et al. (2014a) ('HAPLO 22' on Fig. Ch5-02) at the base of Clade B was amplified from 5/20 *Asellus* spp. collected from the River Avon for this study. As for *C. gammari*, a focused sampling effort targeting isopods of this genus and its close relatives may present an opportunity to further characterise this species and offer greater insight into its phylogenetic affinities.

The amplification of haplosporidian sequence types from soil samples represents the first incidence of haplosporidians outside of aquatic environments which has been confirmed by sequence data. As previously noted, haplosporidian species have been described from non-aquatic invertebrates previously, namely *Haplosporidium melighethi* from the rape blossom beetle *Meligethes aeneus* (Lipa & Hokkanen, 1991), however only low resoltion microscopy images are available for this species, and no molecular data, and it has not been reported since its original publication. This study also provides the first molecular evidence of haplosporidians in insects, with a number of sequence types amplified from Hempitera larvae collected from the River Avon, UK and California Lake in Surrey, UK. It is therefore possible that haplosporidians infect a wider range of invertebrate taxa than previously acknowledged.

No terrestrial invertebrates were collected and screened as part of this study, however it is now important that an effort is made to link this novel terrestrial sequence diversity with host organisms. Gastropods, annelid worms and small crustaceans (amphipods and isopods), are all hosts of haplosporidian parasites

in aquatic environments, are also common in terrestrial environments. A better understanding of the diversity and distribution of haplosporidians in terrestrial hosts, which may or may not include these same groups or even insects, will offer great insight into morphological diversity of the group, as well as host preferences and the life-cycle strategies employed by haplosporidians.

Chapter Six:

An Updated Mikrocytid Phylogeny: Progress and Challenges in a Divergent Group of Microcell Parasites

Georgia M. Ward, Catherine Troman, Céline Garcia, Frederico Batista, Chantelle Hooper and David Bass

Chapter Summary

This chapter builds upon involvement in a previous eDNA-based survey of mikrocytid diversity (see Appendix II), this time with an emphasis on screening bivalve tissue and bivalve-associated incubation and water samples to explore the PCR-determined association of both known and novel mikrocytid diversity with host species, individuals and tissues. The majority of characterised mikrocytid species are pathogens of the Pacific oyster *Crassostrea gigas*, a species favoured for aquaculture activity in Europe and increasingly abundant on the shoreline in the UK as an invasive species. Other bivalve species – cockles *Cerastoderma edule* and mussels *Mytilus edulis* were also screened by PCR, with the aim of establishing whether either of these species are likely to harbour novel mikrocytid diversity, or act as an alternative host or environmental reservoir for known species. A novel lineage strongly associated with *M. edulis*, and amplified from a large proportion (30%) of sampled mussels, is identified, though has yet to be characterised by microscopy. All novel sequence data is placed into a phylogenetic context, along with extended sequence types for two recently described *Mikrocytos* species infecting wedge clams *Donax trunculus* in France (in collaboration with Ifremer), to provide the most complete phylogeny of the order to date.

Consideration is given to the significance of the presence of *Mikrocytos mackini,* a pathogen of *Crassostrea gigas*, detected in European waters for the first time. The parasite is known to be associated with severe mortalities on the Pacific coast of Canada, and until now has not been detected in bivalve-associated samples far outside of this geographical range.

Author's Contribution

Georgia Ward performed all DNA extractions, PCR screens and cloning of environmental DNA and invertebrate samples, with the exception of cockles and mussels collected in the Dart estuary, and water and plankton samples from Mumbles Pier. GMW amplified the novel mikrocytid lineage from *Mytilus edulis*, and designed, optimised and applied the specific primer set used to screen mussels and environmental samples for the presence of the parasite. GMW also performed all phylogenetic analyses.

Introduction

Mikrocytids are microcell parasites within the class Ascetosporea (Rhizaria, Endomyxa, Ascetosporea, Mikrocytida) best known for their effects as pathogens of oysters. *Mikrocytos mackini* is the causative agent of Denman Island disease (mikrocytosis), a major cause of mortality in the Pacific oyster *Crassostrea gigas* on the Pacific coast of Canada, and until very recently was the only fully characterised mikrocytid species. Since 2014 a number of mikrocytid species have been formally described, including *M. boweri* infecting the Olympia oyster *Ostrea lurida* in British Columbia, Canada (Abbott et al., 2014a), *M. mimicus* infecting *C. gigas* in Norfolk, UK (Hartikainen et al., 2014b) and *M. donaxi* and *M. veneroïdes*, both parasites of wedge clams *Donax trunculus* in France (Garcia et al., 2018). *Paramikrocytos*, the only other genus within the order, was described infecting juvenile edible crabs *Cancer pagurus* in the south-west UK (Hartikainen et al., 2014b). *Paramikrocytos canceri* is currently the only species assigned to the genus, and is the first mikrocytid species to be described from a non-bivalve (crustacean) host. Concurrently to the publication of Hartikainen et al. (2014b), Ramilo et al. (2014) reported a parasite of Manila clams *Ruditapes philippinarum* which is morphologically similar to *M. mimicus*, though with a number of differences, particularly cell size and the shape and position of the nucleus in the cell. Unfortunately due to the timing of both publications sequence data for *M. mimicus* were not available to Ramilo et al. (2014) and no comparisons could be drawn, however comparisons of now-published overlapping 18S sequence data shows the two to be highly similar (>99%; 934/938 identical sites), and so it seems likely that these are the same species. The ability to infect more than one host species is not unusual for mikrocytids, and in fact the type species, *M. mackini,* has been found to be

able infect not only *C. gigas* but other oyster species, including *O. edulis* and *O. conchaphila* on the Atlantic Coast of Canada (Bower et al., 1997), and more recently *C. sikamea* in Humboldt Bay, California, US (Elston et al., 2012).

The taxonomic affiliations of mikrocytids remained uncertain for nearly three decades following the discovery of *M. mackini*, however phylogenomic analyses suggested an affiliation with the Ascetosporea as sister group to Haplosporida, parasites of aquatic invertebrates including molluscs, crustaceans and annelid worms (Sierra et al. 2016). The order Mikrocytida was erected within Ascetosporea by Hartikainen et al. (2014b) following multigene phylogenetic analyses with greater taxon sampling of both haplosporidian and mikrocytid taxa.

Mikrocytids are among the most divergent eukaryotes currently known, and as such the generation of 18S sequence data for mikrocytids has been challenging. Most “general” eukaryote primers are too conserved to amplify mikrocytid lineages, though a number of *Mikrocytos*-targeted primer sets have been developed (Abbott et al., 2011; Ramilo et al., 2014). In order to generate sequence data for ribosomal RNA and a number of other genes of *Paramikrocytos canceri,* Hartikainen et al. (2014b) used metagenomic shotgun sequencing of heavily infected antennal gland tissue from the host *C. pagurus*.

Based on these data, and all mikrocytid sequence data available as of December 2012, Hartikainen et al. (2014b) developed a mikrocytid-targeted, nested primer set, and published the results of PCR screens of 511 environmental samples (marine, freshwater and terrestrial) and 425

invertebrate-associated samples including tissue, incubation filters and associated sediments (See Appendix II). While the amplification of mikrocytid sequence types from environmental samples was rare, especially when compared to other ascetosporean orders such as Haplosporida (Hartikainen et al., 2014a; Chapter Five) and Paradinida/ENDO3 (Ward et al., 2018), a total of six novel, uncharacterised sequence types were detected, one falling within the existing genus *Mikrocytos* and the remaining five forming three novel clades found in marine water ('clade a'), brackish and freshwater environmental samples ('clade b') and marine invertebrate tissue ('clade c').

All known mikrocytid species infect hosts which are of some significance to aquaculture activities. A greater understanding of the diversity and phylogeny of the group, its geographic distribution and host range, is important in elucidating life-cycles, and is imperative in the development of robust diagnostics and biosecurity protocols, and understanding potential environmental reservoirs and vectors of mikrocytid diseases. In this chapter, the same nested, group-specific primer set of Hartikainen et al. (2014b) is used to screen a wider range of environmental samples, as well as tissues of known host groups in locations where mikrocytids have not previously been detected. The sequences of key *Mikrocytos* taxa are extended to provide overlapping data for a greater number of species, strengthening the phylogeny of the order. Finally, sequence data is generated for a potential mikrocytid parasite of the blue mussel *Mytilus edulis* in the Tamar estuary, Cornwall, UK.

Materials & Methods

Sample Collection and Processing

Samples of crab larvae, sorted to family level, and bulk zooplankton (including invertebrate larvae and copepods) were collected by the Cefas Endeavour close to the Western Channel Observatory, English Channel, UK, and provided by Paul Bouch at Cefas Lowestoft. DNA was extracted using the DNEasy Blood and Tissue Kit (Qiagen, Germany).

Marine and freshwater environmental samples and invertebrate tissues were collected from locations in Florida, including the Gulf and Atlantic Coasts, in June 2014, and DNA extracted as in Ward et al. (2016).

Mussels *Mytilus edulis* (n = 150) and water samples were collected from Cremyll Ferry on the Tamar estuary, UK in June 2013. A second batch of mussels (n = 156) was collected from the same site in July 2013. All samples were preserved and processed as in Ward et al. (2016). Additional sampling took place in September 2016 and April 2017 at the same site, this time collecting *M. edulis* and feral *Crassostrea gigas*. No aquaculture activity was recorded at this site.

Bulk water samples (n = 12, volume = 10 L each) and sediment samples were taken at high and low tide on the River Dart estuary near Dittisham, Devon in September 2016 and in April 2017 at a site of active *Crassostrea gigas* aquaculture activity. Pacific oysters *C.* were collected from trestles (n = 50) and a large feral population (n = 50), and cockles *Cerastoderma edule* (n = 50) and

M. edulis (n = 50) were collected from the same site on the same occasions. All bivalves were rinsed before incubation in batches of 10-20 in 1 L artificial seawater (ASW). Bivalves were removed from the water after 1 hour and 100 ml of ASW syringe-filtered through 24 µm Whatmann GF/F filters (GE Healthcare, USA). Filters were then fixed in 100% molecular-grade ethanol. All other samples were processed for molecular work and, for the bivalves, histopathology and transmission electron microscopy (TEM) as described in Ward et al. (2016). Bulk water samples (n = 3, 10 L) were collected at high tide at Noss Mayo on the River Yealm estuary, Devon, UK in September 2016 and processed as in Ward et al (2016).

Ethanol-fixed samples of *Donax trunculus* infected with *Mikrocytos donaxi* and *Mikrocytos veneroïdes* were provided by Isabelle Arzul and Céline Garcia at Ifremer, and DNA extracted using the Qiagen Blood & Tissue kit.

PCR & Sequencing

The sequences of all primers used in this study are listed in Table Ch6-T01. All samples were screened by PCR for the presence of mikrocytid sequence types with the nested primer set and cycling conditions of Hartikainen et al. (2014b). Amplicons from water samples were pooled per site and size fraction, and clone libraries constructed using the StrataClone PCR cloning kit (Agilent Technologies, Santa Clara, CA, USA). 8 colonies were picked per library, and unidirectionally Sanger sequenced using the M13R primer. Amplicons produced from invertebrate tissues were sequenced directly using the Mik868f primer.

In order to extend the 18S sequence reads of *Mikrocytos donaxi* and *M. veneroïdes*, PCRs were performed using primers Mm18SF1 and

Mikrocytall1366r. All PCR reactions were performed in 20 µl volumes consisting of 1X Promega Colourless Buffer, 2.5 mM $MgCl_2$, 0.4 mM dNTPs, 0.5 µM each primer, 0.2 mg bovine serum albumin (BSA), 0.5 U GoTaq G2 (Promega, USA), and 1 µl template DNA. Cycling conditions consisted of 5 minutes denaturation at 95°C, followed by 40 cycles of 1 min denaturation at 95°C, 1 min annealing at 58°C and 2 min extension at 72°C, and a final extension at 72°C for 12 min. Amplicons were visualized on 1% agarose-TAE gels stained with GelRed (Biotium) and bidirectionally Sanger sequenced using both amplification primers.

In the course of this study, a divergent mikrocytid lineage was amplified unintentionally as a non-specific product of a microsporidian primer set from a number of mussel *Mytilus edulis* tissues collected in the Tamar estuary. A specific primer set was designed targeting this lineage. PCR reaction mixtures were as above, and used primers Mikrosporo338f and Mikrosporo649r. Cycling conditions consisted of an initial denaturation at 95 °C for 5 minutes, followed by 35 cycles of 1 min denaturation at 95 °C, 1 min primer annealing at 60 °C and 1 min extension at 72 °C. This was followed by a final extension step at 72 °C for 10 min. This primer set was used to screen *M. edulis* digestive gland tissue and marine environmental DNAs.

Phylogenetic Analyses

Mikrocytid sequence data generated from zooplankton (200-2000 µm) and water samples (0.45 - 200 µm; 20 L total volume) collected near Mumbles Pier, Swansea, Wales monthly between September 2017 and January 2018 were provided by Frederico Batista of Swansea University/Cefas.

All newly-generated mikrocytid sequence data was aligned with sequence data previously generated by Hartikainen et al. (2014b) and all full-length mikrocytid sequence types available on NCBI GenBank in August 2018 using MAFFT version 7 (e-ins-i algorithm)(Katoh & Standley, 2013). The mikrocytid sequence type generated from *M. edulis* was excluded from this phylogenetic analysis because there was no significant overlap between this sequence and those generated using the primer set of Hartikainen et al. (2014b).

The resulting alignment was refined manually and analysed using Maximum Likelihood (ML) in RaxML BlackBox version 8 (Stamatakis, 2014) (Generalised time-reversible (GTR) model with CAT approximation (all parameters estimated from the data); an average of 10,000 bootstrap values was mapped onto the tree with the highest likelihood value). A Bayesian consensus tree was constructed using MrBayes v. 3.2.5 (Ronquist et al., 2012). Two separate MC^3 runs with randomly generated starting trees were carried out for two million generations each with one cold and three heated chains. The evolutionary model applied included a GTR substitution matrix, a four-category autocorrelated gamma correction and the covarion model. all parameters were estimated from the data. Trees were sampled every 1000 generations and the first 500,000 generations were discarded as burn-in (trees sampled before the likelihood plots reached stationarity) and a consensus tree was constructed from the remaining sample. Phylogenetic analyses were then repeated using all full-length mikrocytid sequence types and the novel sequence generated from *M. edulis*.

Table Ch6-T01. Sequences of primers used in this study to amplify mikrocytid sequences from environmental and organismal samples. 'Application' indicates the intended specificity and use of these primers in this study.

Primer	Application	Reference
Mik451f: GCCGAGAYGGTTAAWGAGCCTCCT Mik1511r: CCTATTCAGCGCGCTCTGTTGAGA Mik868f: GGACTACCAGWGGCGAAAGCGCCT Mik1341r: TGCATCACGGACCTACCTTWGACC	General Mikrocytida, used to screen all environmental and invertebrate DNA samples.	Hartikainen et al. (2014b)
Mm18Sf: GACGGCAGGAGTATTGTTTGACGA Mikrocytall1366r: GACGGACAGTGTGWACAAGTC	*Mikrocytos* spp., used for the extension of *Mikrocytos* spp. 18S sequences	Abbott et al. (2011) This study
Mikrosporo338f: TTGACCGAGATGGTTATGGGCC Mikrosporo649r: GTCCTGGAACGGTCTGCGA	Basal mikrocytid ex. *Mytilus edulis*, used to screen *M. edulis* tissues and water samples from UK bivalve sites.	This study

Results

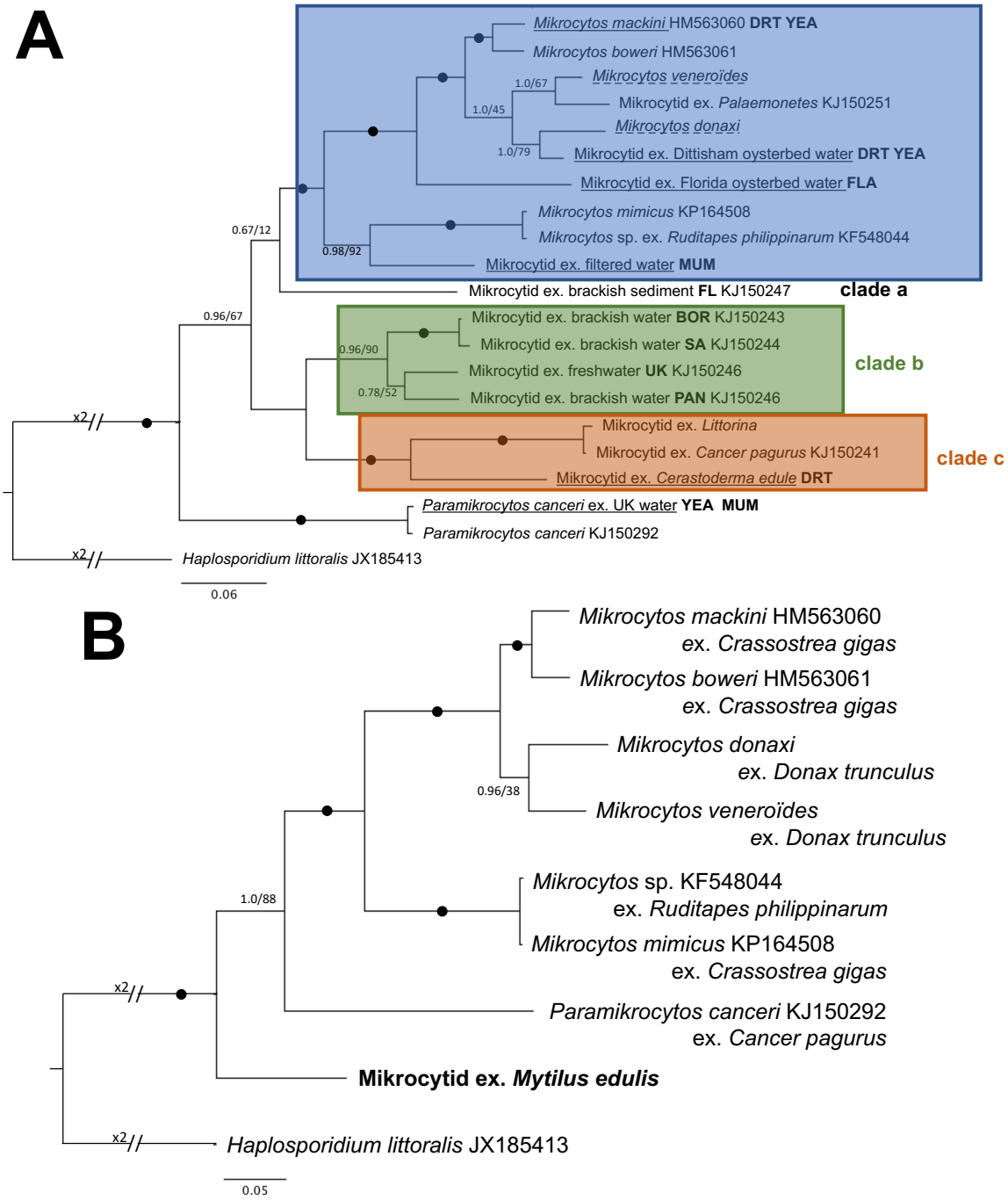

Figure Ch6-01. Bayesian phylogenetic analyses of 18S rDNA sequences of mikrocytids. **A.** All mikrocytid amplicon sequences generated from screens of environmental and invertebrate samples, with all overlapping sequence mikrocytid sequence data. Lineages amplified in this study are underlined, and those extended as part of this study underlined with dashed lines. Codes indicate geographical provenance: BOR = Borneo; DRT =Dittisham (Dart estuary, UK); FL = Fleet lagoon (Weymouth, UK); FLA = Florida (USA); MUM = Mumbles Pier (Swansea, UK); NC = Newton's Cove (Weymouth, UK); PAN = Panama; SA = South Africa; YEA = Noss Mayo (Yealm estuary, UK). **B.** All full-length mikrocytid 18S sequence types including a novel, basal mikrocytid sequence type which does not overlap with all other amplicon data. Circles on nodes indicate maximal support in both analyses, otherwise values indicate Bayesian Posterior Probability/Maximum Likelihood Bootstrap values.

All novel sequence diversity identified in this study falls within previously-detected clades (Fig. Ch6-01A), with the exception of a sequence type generated from the digestive gland tissue of mussel *Mytilus edulis* which groups at the base of known mikrocytid diversity (Fig. Ch6-01B). Three novel sequence types, all amplified from water samples, fall within the *Mikrocytos* radiation (shaded blue on Fig. Ch6-01A), and one further novel sequence type falls within 'clade c' of Hartikainen et al. (2014b), shaded orange on Fig. Ch6-01A. No novel sequence diversity falls within 'clade a', represented by a single sequence type in Fig. Ch6-01A, or 'clade b' (shaded in green), known only from brackish and freshwater sample types. Two characterised sequence types, *Mikrocytos mackini* and *Paramarteilia canceri*, underlined on Fig. Ch6-01A, were amplified from water and invertebrate samples collected in the southwest of the UK.

The extended 18S sequences for *Mikrocytos donaxi* and *M. veneroïdes* place these species in a clade sister to *M. mackini* + *M. boweri* with maximal support. This clade also includes uncharacterised sequence types from shrimp *Palaemonetes* tissue, and a novel sequence type detected in water samples collected at an oyster cultivation site on the River Dart estuary, UK ('Mikrocytid ex. Dittisham oysterbed water' on Fig. Ch6-01A).

Screens of marine and freshwater samples collected at various sites in Florida, USA, including both the Gulf and Atlantic coasts (see Ward et al., 2016) (n = 189) produced a single amplicon, from a bulk water sample collected over Eastern oyster *Crassostrea virginica* beds at high tide on the Atlantic coast. This novel sequence, labelled 'Mikrocytid ex. Florida oysterbed water' on Fig. Ch6-01A, groups with strong support within a clade containing sequence types

belonging to *Mikrocytos* spp. oyster parasites. No mikrocytid lineage was amplified from any other sample from Florida, including invertebrate and freshwater samples.

PCR screens of water samples collected at a Pacific oyster aquaculture site near Dittisham on the River Dart estuary, UK, in September 2016 revealed the presence of a novel mikrocytid lineage in water samples collected over oyster trestles at both high and low tide, and an adjacent pontoon. This lineage, labelled 'Mikrocytid ex. Dittisham oysterbed water' on Fig. Ch6-01A, was not amplified from water samples of the same volume collected 50m downstream of the trestles. Digestive gland and gill tissues from 50 *C. gigas* sampled from the trestles and a further 50 *C. gigas* collected from a large feral population present at the site were also screened using the same primer set, but no mikrocytid lineage was detected in any sample. All bivalve (oyster, mussel and cockle) incubation samples screened were negative, as were all *M. edulis* tissues from the Dittisham site.

PCR screens of gill and digestive gland tissues from 50 edible cockles *Cerastoderma edule* collected from the natural population at the Dittisham site on the River Dart produced two mikrocytid sequence types, both from gill tissue. The first of these was a >99% match to the *Mikrocytos mackini* EURL-recommended diagnostic 18S sequence (GenBank Accession AF477623; 470/471 identical nucleotides). This finding was reported to the England and Wales Competent Authority for aquatic disease (the Fish Health Inspectorate). Histology screens of all tissues from this individual did not detect the presence of *M. mackini* or any other *Mikrocytos*-like organism. The second mikrocytid sequence type amplified from cockle gill tissue ('Mikrocytid ex. *Cerastoderma*

edule' in Fig. Ch6-01A) groups with maximal support as sister to two very closely-related sequences amplified by Hartikainen et al. (2014b), and referred to in that paper as 'clade c'. Both of these sequence types were also generated from invertebrate tissues (a *Littorina* sp. gastropod and edible crab *Cancer pagurus*). Histopathology screens of tissues from the cockle from which the sequence was amplified revealed no visible mikrocytid-like life stages in any tissue.

Bulk water (10 L) collected at Noss Mayo on the River Yealm estuary in Devon, UK in September 2016 was PCR-positive for three distinct mikrocytid sequence types: *Paramikrocytos canceri* ('*Paramikrocytos canceri* ex. UK water in Fig. Ch6-01A)*, Mikrocytos mackini,* and the same novel lineage amplified from the Dittisham water samples ('Mikrocytid ex. Dittisham oysterbed water'; all sequence types labelled YEA on Fig. Ch6-01A). No invertebrate tissue or incubation samples were collected from this site, though edible crabs *Cancer pagurus* and shore crabs *Carcinus maenas*, both known to be hosts of *P. canceri* (Hartikainen et al., 2014b; K. Bateman, pers. comm.), were both prevalent at the site. A small number of feral *C. gigas* and wild blue mussels *Mytilus edulis* were also present at the site, but there was no bivalve aquaculture activity in close proximity to the sampling site at the time of collection.

Screens of bulk water (20 L) and plankton samples collected monthly between September 2017 and January 2018 near Mumbles Pier, Swansea, Wales showed two mikrocytid lineages to be present: a *Paramikrocytos canceri* sequence type ('*Paramikrocytos canceri* ex. UK water') was detected in water

samples collected in November 2017, but no other sample. A novel sequence type – 'Mikrocytid ex. filtered water MUM' on Fig. Ch6-01A – was amplified from water samples collected in October, November and December 2017, and groups with strong support as sister to *C. gigas* pathogen *Mikrocytos mimicus*. No mikrocytid lineage was detected in any sample collected in September or January, or in any plankton sample. Similarly no mikrocytid lineages were amplified from any crab larvae or bulk plankton sample collected in the English Channel in July 2013.

Digestive gland tissue from *M. edulis* collected at Cremyll Ferry on the Tamar estuary (see Chapter One, Chapter Two) in summer 2013 were screened as part of a separate study using microsporidian primers ss18f and Micro33R of Ardila-Garcia et al. (2013). A number of the resultant amplicons were unidirectionally Sanger sequenced using primer ss18f. BLAST searches of sequences against the NCBI GenBank database showed 77% sequence identity to *Paramarteilia canceri* (coverage 70%). Specific primers designed to amplify this lineage (see Table Ch6-01) were used to screen all mussels from Cremyll Ferry and Jupiter Point. Prevalence by PCR at both sites was approximately 30%. This sequence was not amplified from water and sediment samples collected at Cremyll Ferry or Jupiter Point, or any other site sampled. Histological screens of PCR-positive specimens did not detect any microcells in any tissues. As this lineage was detected as a result of non-specific amplification using a different primer set, the sequence covers regions V1 to V4 of the 18S gene, and so cannot be directly compared with sequence data generated using the nested general mikrocytid set (which covers regions V6-V8). This sequence was aligned with all full-length mikrocytid sequence types,

and partial sequence types with coverage in these regions. As shown in Fig. Ch6-01B, phylogenetic analyses place this lineage basal to all sequenced mikrocytids with moderate support (Bayesian posterior probability 0.85; Maximum Likelihood bootstrap 98%).

Discussion

Though the mikrocytid primer set of Hartikainen et al. (2014b) is now known to be limited in its ability to detect mikrocytid diversity, it still amplified a broad range of lineages from a number of different sample types. Bulk water samples were collected from two oysterbeds: eastern oysters *Crassostrea virginica* on the Atlantic coast of Florida, USA, and Pacific oysters *C. gigas* on the River Dart estuary in Devon, southwest UK. Two distinct, novel mikrocytid lineages were amplified from these samples, both grouping with *Mikrocytos* spp. sequence types with maximal support in phylogenetic analyses (Fig. Ch6-01A). Though no samples of *C. virginica* could be collected to screen for pathogens, no notable mortalities which could be attributed to parasite infection were known to occur within this population. Similarly no mortalities were noted at the site on the River Dart, and no mikrocytid sequence types were amplified from digestive gland tissue samples from *C. gigas* collected from the site. *Mikrocytos* infections are also known to be more prevalent at low water temperatures (≤ 10 ºC) (Abbott et al., 2014b), and both the Florida and Dart samples were collected in summer (July and September, respectively). It is possible that both parasites were present in the oyster populations, but at such low levels as to be non-pathogenic, and with infected individuals showing only light, focal infections in unsampled tissue types.

Only two novel sequence types were detected in invertebrate tissue in this study. The first of these, amplified from gill tissue of an edible cockle *Cerastoderma edule,* groups at the base of 'clade c' of Hartikainen et al. (2014b), which is comprised of two closely related uncharacterised sequence types also amplified from invertebrate tissues. In each case the sequence type was only amplified once from a large number of samples of each invertebrate, and there is no microscopic evidence of infection for any of these lineages. It is possible mikrocytid DNA was amplified from these tissues as a result of feeding, or passive environmental transfer.

The strongest association between an uncharacterised mikrocytid sequence type and a potential host is undoubtedly the basal mikrocytid lineage amplified from the digestive gland tissues of mussels *Mytilus edulis* collected in the Tamar estuary, UK. Whilst no mussel examined using light microscopy showed any apparent sign of microcell infection, the small cell size and focal nature of low-level mikrocytid infections can make cells difficult to identify, particularly novel species which do not provoke a strong host response. This sequence type was not originally amplified using the mikrocytid-specific primer set used to screen all samples in this study, rather was detected as non-specific amplification using a primer set targeted at a different group. Subsequent repeat attempts to amplify mikrocytid sequence types using the mikrocytid primer set from these same tissues failed, and so it is likely that at least one primer has a number of mismatches to this novel lineage. As a result, it was not possible to include this lineage and all other uncharacterised sequence types in the same phylogenetic analyses as the sequences cover different regions of the 18S

gene, however the phylogeny presented in Fig. Ch6-01B (albeit with reduced taxon sampling) shows that this lineage is clearly distinct from any other lineage detected. In addition to extending the 18S sequence of this basal lineage by primer walking, an alternative method for generating sequence data (for multiple genes) of this basal lineage is the use of metagenomic shotgun sequencing, as proved successful for the generation of sequence data for *Paramikrocytos canceri* when primer-based methods failed. However such methods require heavily infected tissue, which is not available at this time.

A *Paramikrocytos canceri* sequence type was amplified from water samples collected at Noss Mayo on the River Yealm estuary and near Mumbles Pier in Swansea. These findings represent the first time *P. canceri* has been detected in any environmental sample. PCR screens of host *Cancer pagurus* incubations and sediments collected from directly beneath infected crabs on the shoreline revealed that the parasite is abundant in the urine of infected crabs (and so readily amplified from incubation samples), however was not amplified from any sediment samples. PCR screens of bulk water samples (20–40 L) collected at sampling sites in Weymouth, UK, where the parasite is known to be highly prevalent, were also negative, using both general mikrocytid and lineage-specific approaches (Hartikainen et al., 2014b). *P. canceri* has previously been observed infecting *C. pagurus* at sites in Pembrokeshire, south Wales, where prevalence has been shown to vary significantly between seasons (Thrupp et al., 2013). Prevalence was lower in November (approximately 15%), and much higher in early spring, peaking at 55-70% in March. To date, the majority of environmental water samples screened for *P. canceri* were collected during sampling campaigns when prevalence of the parasite in crabs is known to be

highest at the sites sampled, between late April and July. The amplification of *P. canceri* in this study was from water samples collected at the end of summer and throughout winter (the Noss Mayo samples were collected in mid-September, and the Mumbles samples collected monthly between September and January, though *P. canceri* was only amplified from the sample collected in November). At this time, infection prevalence in crabs is expected to be lower, and the discovery of *P. canceri* in the water column may represent the presence of the parasite in an alternative host, for example a planktonic species smaller than 20 µm.

An important finding of this study is the amplification of oyster pathogen *Mikrocytos mackini* from water and cockle samples collected in south-west England. This is the first molecular detection of this parasite in Europe, and indeed outside of the Pacific coast of North America (Garcia et al., 2018). Whilst the apparent presence of *M. mackini* in Europe may be interpreted as alarming, it is worth noting that no infection was observable in any tissue from the PCR-positive cockle, and no mortalities were reported from any bivalve species at any site on the Dart or Yealm estuaries. *M. mackini* is considered a major pathogen of *C. gigas*, a species exploited for aquaculture globally due to its large size, fast growth rate and apparent resistance to a number of diseases, including *Haplosporidium nelsoni*, *Marteilia refringens* and *Bonamia ostreae*, to which other oyster species are vulnerable. Despite the now almost pan-global distribution of *C. gigas*, mortalities attributed to *M. mackini* are limited to a fairly small geographic range along the Pacific coast of Canada and, more recently, the northwest United States (Abbott et al., 2011; Elston et al., 2012). Only a single report of what Bower et al. (1997) refer to as “structures that appear to be

M. mackini" has ever been made in the native range of the oyster, in Matsushima Bay, Japan, in the 1960s (Numachi et al., 1965). As *C. gigas* is most likely the indigenous host of *M. mackini*, this may suggest that environmental conditions and culturing practices play a large role in the development and pathogenicity of the disease.

Despite seemingly localised mortalities caused by mikrocytosis, the threat of mikrocytid species to global aquaculture must not be underestimated. Neither mortalities nor low-level infections with *M. mackini* have been reported in any host outside of the northwest coast of North America, and any reports of infection in *C. gigas* (or any other host) in new locations should be taken seriously and steps taken to limit translocation of the disease further. Close monitoring of any infected populations, whether mortality is experienced or not, will contribute greatly to our understanding of the effects of environmental conditions and culturing practices on the prevalence and severity of infection.

Though the general mikrocytid primer set was designed to be as inclusive as possible, sequence data for very few taxa – *M. mackini, M. mimicus, M. boweri* and *P. canceri* – were available at the time they were devised, and so it is not surprising that they do not detect sequence types basal to known diversity within the group. Designing a revised, more inclusive primer set which reliably amplifies all mikrocytid sequence types is a priority for future research into the group, and should facilitate the detection of further novel diversity at the base of the order and its distribution in host tissues and the environment. This will also provide the means to amplify complementary sequence data, covering the same regions of the 18S gene, from as many taxa as possible, to allow for more

complete phylogenetic analyses. Despite these limitations, this study reinforces the work of Hartikainen et al. (2014b) and demonstrates the role of targeted eDNA studies in the detection of mikrocytids, both in environmental and tissue samples, and broadens our understanding of the diversity of the group, its hosts and geographic distribution.

The extension of 18S sequences for *Mikrocytos donaxi* and *M. veneroïdes* has allowed comparison between these taxa and all other known mikrocytids for the first time (with the exception of the novel, basal mikrocytid lineage amplified from *Mytilus edulis* tissue in this study). While the taxonomic position of these species was not uncertain, complementary sequence data and longer reads for a greater number of taxa not only produces more robust phylogenies, but is an important step in compiling a database of mikrocytid sequence types with the goal of facilitating the design of the most inclusive general primer sets possible to harmonise the research and diagnostics of a historically challenging taxonomic group which is likely to harbour emerging disease agents and greater novel diversity that is yet to be characterised.

Chapter Seven: Conclusions and Perspectives

Since work began on this thesis in 2014, our knowledge of the Ascetosporea has increased drastically, reflecting not only the growing interest in the group as parasites of importance to aquaculture, but also a marked improvement in the application of sensitive methods, particularly molecular techniques, in the detection and characterisation of both novel lineages and previously known taxa. Similarly the use of eDNA surveys using a broader range of environmental samples than ever before has expanded our knowledge of the ecology, potential host range and diversity of all four characterised orders.

This is perhaps best exemplified by the order Paramyxida. Four new species, all bivalve parasites, have been described in the past five years: *Marteilia cochillia* (Carrasco et al., 2013), *Eomarteilia (Marteilia) granula* (Itoh et al., 2014), *M. octospora* (Ruiz et al., 2016) and *M. pararefringens* (Kerr et al., 2018). Further novel host-parasite interactions have been confirmed by complementary light microscopy and sequence data, such as the involvement of the polychaete *Nephtys australiensis* in the life cycle of *Marteilia sydneyi* (Adlard & Nolan, 2015), and the infection of velvet crabs *Necora puber* with *Paramarteilia* sp. (Ward et al., in prep). Chapter One produced 18S sequence data for *Paramyxa nephtys* for the first time, and used *in situ* hybridisation techniques to unambiguously link this with histology and electron microscopy of the parasite in host tissue. The PCR screens presented in this same chapter indicate a number

of novel host-parasite interactions, for example the strong association between *Paramyxa* sequence types and mussels *Mytilus edulis* and oysters *Ostrea edulis* in the Tamar estuary, Cornwall, UK, and yet more are coming apparent, with the very recent finding of *Paramarteilia* 18S sequence types in cockles *Cerastoderma edule* tissue in the Tamar estuary and the Dyfi estuary, Ceredigion, Wales (Ward et al., in prep; J. Ironside, Pers. Comm.).

Environmental Diversity and Distribution of Ascetosporea

This study revealed significant novel sequence diversity, much of which is uncharacterised. Without complementary morphological data, it is impossible to contextualise these sequence types and begin to understand inter- and intra-specific sequence diversity. Such information is required to develop robust molecular diagnostic methods.

Paradinida remains the most poorly characterised of the ascetosporean orders for a number of reasons. Interest in and awareness of the group has been slow to develop following the description of *Paradinium* spp. by Chatton nearly 100 years ago (Chatton, 1920), and paradinids are rarely encountered in studies of zooplankton, thought to be a result of the strong seasonality of infections and a lack of research attention to copepod endoparasites (Skovgaard & Saiz, 2005). Parasites of small planktonic invertebrates are understudied in comparison to those infecting larger or commercially exploited aquatic hosts, or hosts of conservation concern. It should be noted, however, that while paradinids are largely known as parasites of copepods, at least one species within this radiation is a parasite of a larger – and economically significant – crustacean

(the spot prawn *Pandalus platyceros*), and so the relevance of the group to aquaculture cannot be discounted.

The phylogenies presented in Chapter Four establish the ubiquity and diversity of paradinid lineages in littoral and coastal samples, and indicate that there is a large radiation of novel sequence diversity within the order yet to be characterised in terms of their morphology and ecology. Zooplankton are key organisms in aquatic ecosystems and play an important role in the control of primary producers (phytoplankton), as well as having their own place in aquatic food chains as an energy source for larger zooplanktonic species, and species such as fish and cetaceans. The *Tara* Oceans expedition revealed that the majority of interactions between taxa in planktonic samples are symbiotic (Lima-Mendez et al., 2015), though it remains to be determined the extent to which these interactions are mutualistic, commensal or pathogenic. An important next step is to understand the host range of the group, and their impact upon their host's grown, reproduction and mortality. Efforts are already underway to collect and preserve planktonic copepods for complementary microscopy (histopathology) and molecular work, as has been shown to be effective for linking morphology with sequence data for other ascetosporean species.

Another important outcome of Chapter Four is the amplification of further novel sequence diversity at the base of Ascetosporea. Prior to this study, the closest characterised relatives of Ascetosporea were the free-living giant amoebae *Gromia* and *Filoreta*, however the targeted primer approach taken in this chapter amplified novel sequence diversity within ENDO-2 (previously known only from deep-sea samples, and whose inclusion within the Ascetosporea is

uncertain), as well as revealing novel lineages ENDO-6 and ENDO-8 in coastal water and sediment samples, respectively. These three clades, particularly ENDO-2 and ENDO-6, are of great interest because phylogenetically they fall between taxa which are free-living and those which are parasitic. Determining the morphology and life history of these lineages, for example using techniques such as fluorescent *in situ* hybridisation (FISH), as has already been used effectively for other rhizarian clades (e.g. Aquavolonida, Bass et al., 2018), would be of great interest not only due to the insight it would offer into the evolutionary history of an important group of aquatic parasites, but also furthering understanding of the morphological and ecological diversity of rhizarian lineages.

Chapter Five revealed the presence of significant uncharacterised haplosporidian sequence diversity in freshwater and terrestrial soil sample types. While the discovery of haplosporidians in freshwater environments was not unexpected, the extent of this diversity (shown in Fig. Ch5-01) and the geographic distribution of sequences in freshwater clades in samples collected across Europe, the United States and Borneo is surprising. Freshwater amphipods, snails and bivalves are already known as hosts of *Haplosporidium* spp. (*H. diporeiae, H. pickfordi* and *H. raabei* respectively), and haplosporidian sequence types were commonly detected in amphipods and snails screened by PCR in this study.

The amplification of haplosporidian sequence types from terrestrial soils is more surprising, and certainly warrants further study. Haplosporidian host groups including amphipods, gastropods and annelids are all common in terrestrial

ecosystems, and so PCR screens of these taxa are a logical next step in uncovering potential hosts. It is important that subsequent research into the hosts of these 'non-marine' haplosporidian lineages pairs molecular characterisation with histopathology and *in situ* hybridisation, to facilitate the comprehensive characterisation of these parasites and increase our understanding of the morphological diversity of the group.

Environmental DNA surveys also offered insight into the sequence diversity and distribution of Paramyxida and Mikrocytida, though at first glance it may seem these surveys were less 'successful'. No large radiations of novel sequence diversity were detected in eDNA screens for either order, however screens of potential host tissues and host-associated samples, for example water samples collected over oyster beds, reveal promising associations. Two novel *Mikrocytos* lineages and two as-yet uncharacterised paramyxid lineages (PARAM-1 and PARAM-2) were all amplified from water samples collected from beds of *Crassostrea* spp. in the UK and Florida.

The apparent absence of paramyxid and mikrocytid sequence types in the water column may also offer insight into the modes of transmission of these parasites, and their seasonality. Ongoing PCR screens of various sample types for the presence of paramyxids have revealed a strong link between *Paramarteilia* sequence types and larval stages of crabs sorted from bulk zooplankton samples (Ward et al., in prep). This is not surprising, given that *Paramarteilia canceri* has been observed infecting the ovocytes of its host *Cancer pagurus* (see Chapter One), and so it is likely that the parasite is vertically transmitted. If this is the case, the apparent scarcity of *Paramarteilia* in

the water column may be a result of different dispersal and transmission strategies to those adopted by other ascetosporean lineages. Characterisation of taxa prevalent in the water column and the further elucidation of ascetosporean life-cycles is an important step in interpreting the significance of environmental DNA findings.

eDNA studies to date have focused largely on littoral and coastal marine, and freshwater samples. Sequence types belonging to Haplosporida, Paramyxida and Paradinida can also be found in the 18S V9 metabarcoding data generated from oceanic plankton samples collected by the *Tara* Oceans Expedition (C. Berney, pers. comm), suggesting our knowledge of ascetosporean diversity continues to be biased by sample type. While no mikrocytid sequence types were detected in the *Tara* data, this is most likely due to their highly divergent sequence types not being amplified by the general eukaryote primer sets used. Hartikainen et al. (2014b) screened a small number of planktonic copepod samples collected in northern and southern hemisphere oceans, and detected the oyster pathogen *Mikrocytos mackini* in copepods from the South Atlantic. The diversity of Ascetosporea in oceanic and planktonic samples, and indeed in other high throughput sequencing datasets, is worthy of further investigation, and offer hugely valuable insight into the distribution of the group.

Though the eDNA surveys are useful tools for the detection of sequence diversity in environmental sample types, there are a number of important limitations of such techniques. A lack of complementary morphological data prevents any certain statements being made about the life-stage or viability of cells potentially associated with the sequence detected, and so life-cycle

information cannot easily be inferred from such data. Many parasites show seasonality in their infection prevalence and life-cycle stages, and so the apparent absence of specific sequence types from environmental samples may not necessarily reflect their occurrence in the water column or sediment at other times.

Many of the sample sets used in this study were not collected with the specific goal of screening for Ascetosporea, particularly datasets from Florida and Borneo, and the soil samples screened for haplosporidians in Chapter Five. As such, results from these datasets should be interpreted with caution. In these cases, as is also true for other datasets, the samples collected represent only a single point in time, and so cannot be taken as being representative of all Ascetosporea present in these geographic regions. Water samples collected at this site were also processed very differently than those collected during sampling campaigns tailored to Ascetosporea, with only larger size-fractions filtered through 50- and 20-μm meshes. This too introduces bias to the PCR screening methods employed, since smaller size-fractions will have been lost during the filtering process.

No sample set utilised in this study allowed for the opportunity to gain insight into the changes in the presence or abundance of any lineage in the water column or sediment over time. To achieve this, different sampling strategies and screening protocols are required. Finely-timed, frequent and tailored sampling methods, ideally targeting specific species or lineages, are required, and molecular screening methods should be adapted. The use of broad PCR primers allows for the detection of a wider diversity of sequence types, however

these strategies also lead to PCR bias and so specifically targeted PCR or qPCR assays will offer greater, more reliable insight.

Complementary Techniques for Parasite Characterisation

Though the detection of novel sequence types in environmental samples has given us great insight into the distribution and diversity of Ascetosporea, it should now be a priority to identify hosts of these lineages and link this sequence data with morphology. This will advance our understanding of the relationships between host preference and phylogenetics, and aid in the elucidation of ascetosporean lifecycles.

Chapter Three demonstrates clearly the efficacy of host-based molecular approaches for the detection and characterisation of haplosporidian parasites. Though haplosporidian plasmodia had been observed infecting *M. edulis* in the Tamar estuary prior to this study (J. Bignell, pers. comm), no pathology was associated with the presence of the parasite and no resources allocated for their molecular characterisation. No spore stages were observed for either *Minchinia mytili* or *Haplosporidium* sp., however the populations sampled in each case were studied over a short time and so it is possible that sporulation occurs at a different stage of infection. Nevertheless, the molecular probes developed for each parasite, which should be applied as a diagnostic PCR and *in situ* hybridisation, paired with traditional histopathology, can facilitate the detection of parasite life-stages within host tissues, and may reveal mature parasite stages in other *M. edulis* samples or in a different definitive host. In this way these tools may aid in the elucidation of the life-cycle and seasonality of these parasites.

An enduring problem in haplosporidian phylogenetics is the apparent paraphyly of the genus *Haplosporidium.* This is unlikely to be resolved until the type species of the genus, *H. scolopli*, can be sequenced, or a suitable neotype designated. A large number of the described *Haplosporidium* species discovered in the early 20th Century are not characterised molecularly, and targeted efforts to sequence key taxa may pair morphology with as-yet uncharacterised sequence data, so offering some valuable and much lacking insight into the phylogenetic patterns of haplosporidian hosts.

Chapter One shows that through the targeted sampling of the polychaete *Nephtys caeca*, known host of the unsequenced paramyxid *Paramyxa nephtys*, it was not only possible to generate 18S sequence data for this lineage, but that this sequence type had previously been detected in water samples collected in the same geographic region. As a result, 18S sequence data is now in fact available for all but three of the described paramyxid species, allowing for a comprehensive phylogeny of the order and providing a robust framework into which to place novel findings.

Molecular Approaches for Parasite Detection and Disease Diagnosis

As discussed above, molecular approaches have proven effective at detecting a broad range of novel ascetosporean 18S sequence diversity within a range of sample types. Molecular techniques, particularly those which allow complementarity between molecular and morphological approaches, such as *in situ* hybridisation, can also improve detection of early parasite stages and

newly-established infections which are yet to elicit a host response, as demonstrated in Chapter Two for the paramyxid *Marteilia pararefringens* in host *Mytilus edulis*. The work presented in this chapter shows that PCR using DNA templates alone is not appropriate for accurate disease diagnosis, with the majority of 'positive' samples showing no transcriptional activity of the parasite in cDNA from the same tissues. Additionally *in situ* hybridisation of tissues PCR-positive using a cDNA template was shown to be more effective than standard histology protocols alone for detecting nascent infections in mussel tissue. The detection of low-level, difficult to detect infections, particularly in hosts traded as aquaculture commodities between different geographic regions, is an important step in preventing the unintentional distribution of pathogens to new areas. *Marteilia pararefringens* may not currently be considered a major cause of mortality in any bivalve host, however this does not reduce the importance of studying its impact on mussels, both at the individual and population level, and elucidating its life cycle both within and outside the bivalve host. Not only will this lead to greater understanding of the differences between *M. pararefringens* and *M. refringens*, but also increases our knowledge of paramyxid life cycles, transmission strategies and alternative hosts.

PCR diagnostics may offer faster, more sensitive detection of pathogens within host tissue, but there are significant limitations. Infections may be highly focal, particularly in the early stages, and in some cases may occur in tissue types other than those in which the pathogen is usually observed (as demonstrated by the observation of *Marteilia pararefringens* in the gills of *M. edulis* in Chapter Two). Targeting a single tissue type for DNA (or RNA) extraction and PCR may not detect such infections, while traditional histopathological techniques often

include sections of whole animals and so offer an increased opportunity for the detection of focal or “unusual” infections. Molecular approaches in which DNA is extracted from a homogenate of all tissues may also provide misleading results, as parasite life stages present only in a single tissue may be effectively diluted and require very sensitive PCR with a larger number of amplification cycles to be effectively detected.

Another danger of PCR diagnostics is a lack of amplification as the result of PCR inhibition. Poorly-preserved tissues and carry-over of contaminants during the extraction process may result in false negatives, and if molecular methods are not paired with complementary microscopic methods, infections may be missed.

Phylogeny and Legislation

Sequence data including at least partial 18S and, in most cases also the first internal transcribed spacer (ITS1) region, are available for nearly all characterised paramyxid species. This has allowed for the comprehensive paramyxid phylogeny shown in Chapter One. While this phylogeny may appear laterally compressed, especially when compared to phylogenies of other ascetosporean orders, it is important to note that all sequence types used in the phylogenetic analyses are unique, and high 18S similarity between phenotypically very different taxa is not uncommon for protists (Boenigk et al., 2012). Non-coding ITS1 region sequences show much greater sequence variation between species, and so are a more suitable marker for discrimination between closely related paramyxid species with identical 18S sequences and

very similar morphology, for example *Marteilia refringens* and *M. pararefringens* (Kerr et al., 2018; Appendix II) and *Paramarteilia orchestiae* and *Paramarteilia* sp. infecting velvet crabs *Necora puber* (Ward et al., in prep).

It is important to prioritise the generation of whole genome and transcriptome data for paramyxid species (and other Ascetosporea) in order to facilitate phylogenomic approaches to elucidating paramyxid relationships, relationships between the ascetosporean orders, and strengthening our knowledge of the position of Ascetosporea within Rhizaria.

The imperative for an improved, high-resolution understanding of paramyxid phylogeny is the importance of robust, readily applicable molecular markers for pathogen detection in aquaculture. The majority of hosts of characterised paramyxid species are of commercial value, and as such pathogenic species – including *M. refringens* and, until recently, *M. sydneyi* – are subject to World Organisation for Animal Health (OIE) and European Union legislation, while close relatives are not. Where a pathogen influences trade, whether on local, national or international scales, protocols used for disease diagnosis must be rapid, sensitive and specific, so as to minimise associated economic losses. Traditional histopathology will always have a place in aquatic disease diagnosis and characterisation, however increasingly molecular diagnostics are taking on an important role. Sequencing of diagnostic markers (in this case ITS1), as recommended for discrimination between *M. refringens* and *M. pararefringens* (which is not subject to legislation), is an effective method of ensuring legislation is applied only in cases where it is necessary, and conversely ensures that full legislative action is taken where needed.

Future Challenges for Ascetosporean Research

Perhaps the most obvious outstanding issue in the research of Ascetospora concerns the phylogenetic relationships between the orders. Little is known about these relationships, and there are few morphological clues as to potential affiliations. While a considerable amount of sequence data is now available for each order, most is amplicon sequence data generated by group-specific primers, where it has not always been possible to target the same variable regions of the 18S gene to allow comparison across orders, and the use of such short sequencing reads to resolve relationships is not ideal. Phylogenomic analyses have previously been used to confirm the inclusion of Mikroctida within Ascetosporea (Sierra et al., 2015), and multigene analyses using a greater (though still very small) number of relevant taxa confirmed a relationship between Mikrocytida and Haplosporida. Synthesis of sequence data for a number of genes for key taxa across the class will offer insight into the relationships between the groups, as well as strengthening the phylogenetic placement of Ascetosporea within Rhizaria.

Interactions between Ascetosporea and their hosts are also poorly understood, and the generation of transcriptome data from infected tissue, particularly for species impacting commercially important hosts, is a key step to understanding host-pathogen interactions.

As is generally the case for parasites relevant to aquaculture, new species descriptions within Ascetosporea are still concentrated in regions with large, developed aquaculture sectors (particularly Europe and East Asia), where there

are researchers with relevant expertise, and in commercially important hosts. However the majority of described haplosporidian species are not known to infect commercially exploited hosts, and in comparison little is known of the effects these parasites have on their hosts. Many of the host groups of Ascetosporean species play important ecological roles, for instance as filter feeders (e.g. bivalve molluscs) and in the perturbation and oxidation of marine sediments and terrestrial soils (e.g. annelid worms). The impact of parasites as ecosystem engineers is complex, and it is impossible to predict the effects of environmental change or disturbance such as rises in water temperature, extreme weather or anthropogenic influences on aquatic or terrestrial habitats on parasite prevalence, distribution or pathogenicity. A greater understanding of the relationships between Ascetosporea and their hosts, and their geographic distribution within those hosts, may go some way to elucidating these interactions.

This study has played an important role in establishing phylogenetic frameworks into which novel discoveries can be placed. However as demonstrated by the need to redesign “general” paramyxid and mikrocytid primer sets following the discovery of novel, divergent sequence types, molecular methodologies will be required to adapt over time.

This work has also demonstrated the abundance of Ascetsporea in environmental and organismal sample types, and a priority for future research should be to contextualise these findings with tightly-paired molecular and microscopy-baed studies. All four orders focused on in this study were found in every geographic location screened, suggesting a global distribution of

Ascetosporea. The implementation of sampling of similar sample types (water column, sediment, invertebrate tissue), particularly in locations with established and/or expanding invertebrate aquaculture sectors, will facilitate the identification of potential pathogens of economic significance, and allow for the rapid characterisation of emergent diseases, and should be considered a research priority to ensure the sustainability of aquaculture. This statement applies not only to Ascetosporea but to other important parasite groups, whether pathogens of invertebrates, finfish or algae.

Group-targeted PCR approaches were used to great effect in this study, and in future should be paired with increasingly affordable high-throughput short read sequencing platforms (Illumina HiSeq, MiSeq) and newer long-read sequencing platforms such as PacBio and Nanopore, to allow for deeper sequencing of environmental and organismal samples.

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Appendix I: Journal-Formatted Published Papers

Appendix II: Journal-Formatted Published Contributions